

Characterisation of the cellular basis of beta-lactam induced skin and liver injury

This thesis is submitted in accordance with the requirements of the
University of Liverpool for the degree of Doctor in Philosophy by

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Declaration

I declare that the work presented in this thesis is all my own work and has not been submitted for any other degree.

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Manal M. Monshi

Dedicated to
My Husband Abdullah, My Parents, My Parents- In- Law and My Children
(Shahad and Ayman)

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Contents

Acknowledgements	iv
Abbreviations	vi
publications	x
Abstract	xii
1. General introduction	1
2. The role of drug-specific T-cells in piperacillin hypersensitive patients	50
3. The role of T-lymphocytes in cystic fibrosis patients with multiple beta-lactams allergy	87
4. Characterisation of flucloxacillin specific T-lymphocyte responses in drug-induced liver injury	100
5. Studies to investigate mechanisms of flucloxacillin presentation to T-lymphocytes	123
6. Final discussion	140
References	151

Abbreviations

ABC Abacavir

ADR Adverse drug reaction

ALP Alkaline phosphatase

ALT Alanine aminotransferase

AMP Adenosine monophosphate

APC Allophycocyanin

ARDS Acute respiratory distress syndrome

BE Bullous exanthema

BSA Bovine serum albumin

β2m β 2 microglobulin

CCR Chemokine receptor (C-C) motif

CD Cluster of differentiation

CF Cystic fibrosis

CFSE Carboxyfluorescein diacetate succinimidyl ester

CI Confidence interval

cpm Counts per minute

CSA Cyclosporin

CTL Cytotoxic T-lymphocyte

CYP Cytochrome P450 enzyme

DAMP Damage associated molecular pattern

DC Dendritic cell

DHR Drug hypersensitivity reaction

DILI Drug-induced liver injury

DISI Drug-induced skin injury

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

DNCB Dinitrochlorobenzene

DRESS Drug reaction with eosinophilia and systemic symptoms

EBV Epstein-Barr virus

EDTA Ethylenediaminetetraacetic acid

ELISpot Enzyme-linked immunospot

ER Endoplasmic reticulum

ERK Extracellular-signal-regulated kinase

FACS Fluorescence activated cell sorting

FasL Fas ligand

FBS Foetal bovine serum

FITC Fluorescein isothiocyanate

GMCSF Granulocyte-macrophage colony-stimulating factor

GrzB Granzyme B

h Hours

HBSS Hanks balanced salt solution

HEPES hydroxyethyl piperazineethanesulfonic acid

HIV Human immunodeficiency virus

HLA Human leukocyte antigen

Hm Homozygote

Hsp70 Heat shock protein 70

HSS Hypersensitivity syndrome

Ht Heterozygote

IFN γ Interferon- γ

IgE Immunoglobulin E

IL Interleukin

IS Internal standard

ITAM Immunoreceptor tyrosine-based activation motifs

JNK c-Jun N-terminal kinase

LAT Transmembrane adapter protein linker for the activation of Tcells

LCMS/MS Liquid chromatography tandem mass spectrometry

LPS Lipopolysaccharide

LSD Lysergic acid diethylamide

LTT Lymphocyte transformation test

ME Maculopapular exanthema

MHC Major histocompatibility complex

min Minutes

MRM Multiple reaction monitoring

ND not determined

NFAT Nuclear factor of activated T-cells

NHS National Health Service
NK Natural killer
NP Not performed
NRTI Nucleoside reverse transcriptase inhibitor
OR Odds ratio
PAMP Pathogen associated molecular pattern
PBMC Peripheral blood mononuclear cell
PBS Phosphate buffered saline
PE Papular exanthema
PE Phycoerythrin
pi Pharmacological interaction
PKC Protein kinase
RNA Ribonucleic acid
RPMI Roswell Park Memorial Institute
SFC Spot forming cell
SI Stimulation index
SJS Stevens-Johnson syndrome
SLP76 SH2 domain-containing leukocyte phospho-protein of 76kDa
SMX Sulfamethoxazole
SMXNO Nitroso sulfamethoxazole
STAT Signal Transducer and Activator of Transcription
TAP Transporter associated with antigen processing
TCR T-cell receptor
TEN Toxic epidermal necrolysis
Th1 Type 1 helper cell
TNCB Trinitrochlorobenzene
TNF α Tumour necrosis factor- α
TNP Trinitrophenol
TPMT Thiopurine methyltransferase
T_{RM} Tissue-resident memory cells
UK United Kingdom
ULN Upper limit of normal
v/v volume/volume

w/v weight/volume

WHO World Health Organisation

ZAP70 ζ -chain-associated protein kinase 70

Publications

Published papers

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4. Monshi MM, Faulkner L, Gibson A, Jenkins RE, Farrell J, Earnshaw CJ, Alfirevic A, Cederbrant K, Daly AK, French N, Pirmohamed M, Park BK, Naisbitt DJ. Human leukocyte antigen (HLA)-B*57:01-restricted activation of drug-specific T cells provides the immunological basis for flucloxacillin-induced liver injury. *Hepatology*. 2013 Feb; **57**(2):727-39.

5. Jenkins RE¹, Yaseen FS¹, Monshi MM¹, Whitaker P, Meng X, Farrell J, Hamlett J, Sanderson JP, El-Ghaiesh S, Peckham D, Pirmohamed M, Park BK, Naisbitt DJ. β -Lactam antibiotics form distinct haptenic structures on albumin and activate drug-specific T-lymphocyte responses in multiallergic patients with cystic fibrosis. *Chem Res Toxicol*. 2013 Jun 17; **26**(6):963-75.

Abstract

Drug hypersensitivity reactions are a significant cause of patient morbidity and mortality. They are difficult to predict in the clinic and during the drug development process. This is because mechanisms have not been fully elucidated. In fact, recent data using T-cells cloned from hypersensitive human patients have questioned the long-standing hapten concept, which states that a drug must bind irreversibly to protein to initiate a T-cell response. The work described herein was performed to investigate the chemical and cellular mechanisms of β -lactam-induced skin and liver injury and in particular to understand the relationship between drug-protein adduct formation and the activation of antigen-specific T-cells.

β -lactam antibiotics, such as piperacillin, provide the cornerstone of treatment and reduce the rate of decline in lung function in patients with cystic fibrosis, but use is limited by a high frequency of hypersensitivity reactions. Using the lymphocyte transformation test, drug-responsive lymphocytes were found in approximately 70% of clinically diagnosed piperacillin hypersensitive patients. By cloning over 400 antigen-specific CD4+, CD8+ and CD4+CD8+ T-cells, the T-cell proliferative response and cytokine secretion to piperacillin was shown to be concentration-dependent and highly drug-specific. Mass spectrometry revealed irreversible binding of piperacillin to selective lysine residues on albumin and a synthetic albumin conjugate stimulated piperacillin-specific clones via a processing-dependent pathway. These results describe the cellular processes that underlie piperacillin hypersensitivity.

Approximately 20% of hypersensitive patients with cystic fibrosis develop multiple reactions that restrict therapeutic options. To explore the mechanistic basis of multiple β -lactam hypersensitivity, albumin binding profiles and T-cell responses against three commonly prescribed drugs; piperacillin, meropenem and aztreonam, were studied. PBMC responses were characterized using the lymphocyte transformation test and IFN- γ ELISpot. Clones were generated and found to proliferate and release cytokines following stimulation with all three drugs. However, crossreactivity with the different drugs was not observed. Each compound formed distinct haptens with lysine residues on albumin, which may explain the highly drug-specific T-cell response. These data indicate that multiple β -lactam reactions are instigated through priming naïve T-cells against the different drugs.

The role of the adaptive immune system in reactions that target liver has not been defined. For flucloxacillin, a delay in the reaction onset and identification of HLA-B*57:01 as a susceptibility factor are indicative of an immune pathogenesis. Thus, flucloxacillin-responsive CD4+ and CD8+ T-cells were characterized from patients with liver injury. Clones expressed the gut-homing chemokine receptors CCR4 and CCR9 and secreted IFN- γ , Th2 cytokines and cytolytic molecules following drug stimulation. In contrast to the piperacillin clones, flucloxacillin clones were activated with several structurally-related β -lactam antibiotics. Furthermore, naïve CD8+ T-cells from volunteers expressing B*57:01 were activated with flucloxacillin when dendritic cells presented the drug antigen. Activation of CD8+ cells from patients and volunteers was processing-dependent and restricted by HLA-B*57:01, which effectively links the genetic association to the iatrogenic disease.

In conclusion, the studies described herein provide novel insight into the way in which β -lactam antibiotics interact with protein and activate T-cells that are thought to be the ultimate mediators of drug hypersensitivity reactions in skin and liver.

Chapter One

General Introduction

1.1 Introduction	2
1.2 Adverse drug reactions	2
1.2.1 Definition of adverse drug reactions	2
1.2.2 Impact of adverse drug reactions	3
1.2.3 Classification of adverse drug reaction	4
1.3 Immunological reactions	6
1.3.1 Classification of immunological reactions	6
1.4 Immune activation	8
1.4.1 Components of the immune system	8
1.4.2 Antigen processing, presentation and cross presentation	14
1.4.3 Intracellular signalling following activation of TCR	18
1.5 Drug-induced skin injury (DISI)	19
1.5.1 Immune cells in the skin	20
1.5.2 Clinical presentation and mechanisms of DISI	21
1.6 Drug-induced liver injury	25
1.6.1 Immune cells in the liver	25
1.6.2 Mechanisms of DILI	26
1.6.3 Clinical presentation of DILI	27
1.7 The major histocompatibility complex	28
1.7.1 Location and variation	28
1.7.2 MHC class I	28
1.7.3 MHC class II	28
1.7.4 MHC class III	29
1.7.5 HLA nomenclature	29
1.7.6 Mechanisms of T-cell activation by drugs	31
1.7.7 HLA association and adverse drug reactions	36
1.8 Adverse drug reactions without HLA association	44
1.8.1 Piperacillin	44
1.9 Aims of the thesis	49

1.1 Introduction

Immune-mediated reactions to drugs are difficult to predict. They can be exceedingly severe in nature which means they are a major problem for clinicians and researchers in the pharmaceutical industry. It has been reported recently that susceptibility to adverse drug reactions is increased with the possession of particular human leukocyte antigen (HLA) alleles. However were this relates to a highly HLA-restricted drug-specific T-cell response is not yet known.

1.2 Adverse drug reactions

1.2.1 Definitions of adverse drug reactions

The World Health Organisation (WHO) in 1972 defined adverse drug reactions as follows: “A response to a drug that is anxious and unintended and occurs at doses normally used in man for the prophylaxis, diagnosis or therapy of disease, or for modification of physiological function” (WHO, 1972).

However a more comprehensive and relevant definition has been suggested by (Edwards and Aronson, 2000): “An appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medicinal product, which predicts hazards from future administration and warrants prevention or specific treatment, or alteration of the dosage regimen”.

The second definition is more useable as suggested by the authors for the reason that it refers to severe reactions, involves reactions caused by prescription error and involves reactions caused by excipients or contaminants.

1.2.2 Impact of adverse drug reactions

Nearly 3% (Zhang et al., 2012) of new therapeutic compounds will eventually be withdrawn from the market because of safety concerns, mostly in the first two years after approval, in spite of rigorous strategies including pre-clinical and clinical testing.

Adverse drug reactions (ADRs) have been identified as a significant health problem that requires a thorough investigation because of their high incidence and related severity. A prospective study in 2004 found that approximately 6.5% of hospitalizations, more than eighteen thousand patients in the North-West of England, were related to an adverse drug reaction (Pirmohamed et al., 2004). The most prevalent age group were the elderly (median age in non-ADR group = 66; median age in ADR group = 76) and among women (52% in non-ADR group; 59% in ADR group) (Pirmohamed et al., 2004). This is compatible with other data collected from different centres around the UK (incidence of ADRs 20.6/10,000 female patients vs. 12.9/10,000 male patients) (Martin et al., 1998). Lack of reporting in males might be the cause of higher prevalence of ADRs in females. Patients recovered from their ADRs in the majority of cases, but still 2.3% of ADRs were categorised as being the main causality for patient mortality (Pirmohamed et al., 2004). In total, 0.2% of mortalities in hospitalised patients were related to ADRs (Lazarou et al., 1998; Pirmohamed et al., 2004). Based on this analysis, Lazarou et al. proposed that ADRs represent between the sixth and fourth most important reason for death in the USA (Lazarou et al., 1998).

ADRs also have serious economic consequences besides their direct threat to patient health safety. Pirmohamed et al. proposed that ADRs represent a major financial burden as it cost the NHS over four hundred million pounds sterling per annum (Pirmohamed et al., 2004). Moreover, a substantial investment is being lost by pharmaceutical companies following compound withdrawal in the late stage of development. Several estimates suggest that the financial cost of getting a new drug

compound to market can reach up to eight hundred million dollars (DiMasi et al., 2003).

1.2.3 Classification of adverse drug reactions

An array of drugs with different indications has been reported to cause serious adverse drug reactions [Table 1.1]. The prevalence of ADRs varies greatly for different drugs. Reactions can generally be categorised in to two main groups; type A and type B (Park et al., 1998).

Drug	Medical indication	Adverse reaction	Frequency of ADR in study population	Date withdrawn	Reference
Abacavir	HIV	Hypersensitivity	5-8%	Still in use	(Mallal et al., 2002)
Carbamazepine	Epilepsy	SJS/TEN	0.1-0.01%	Still in use	(Vittorio and Muglia, 1995)
Flucloxacillin	Staphylococcal infection	Cholestatic/mixed liver injury	0.0085%	Still in use	(Russmann et al., 2005)
Isoniazid	Tuberculosis	Hepatotoxicity	10-20%	Still in use	(Mitchell et al., 1976)
Rifampicin	Tuberculosis	Hepatotoxicity	<5%	Still in use	(Grosset and Leventis, 1983)
Troglitazone	Diabetes	Hepatotoxicity	1.9%	2000	(Watkins and Whitcomb, 1998)
Warfarin	Anti-coagulant	Bleeding	10-16%	Still in use	(Wysowski et al., 2007)
Ximelagatran	Thromboembolism	hepatotoxicity	7.9%	2006	(Lee et al., 2005)

Table 1.1- Examples of various adverse drug reactions

Type A (on-target) reactions are in general a dose function, resulting in overstated pharmacological outcome. They can be affected by enzyme inhibition and or induction by concomitant therapies, or by metabolism or drug transporters. In the majority of cases reactions can be controlled through adjustment of the dose, or by careful monitoring of medications. The majority of ADRs fall under the category of type A reactions. Type B (off-target) reactions are less frequent, when compared to type A reactions, however they are a common cause of drug mortality/morbidity as a result of their unpredictable and severe nature. Effects are not related to pharmacological actions, reactions are only observed in a minority of exposed individuals. It is extremely difficult to determine the underlying mechanisms behind off-target reactions.

A minority of ADRs cannot be categorised under the umbrella of the above classification therefore the following definitions may also be relevant (Edwards and Aronson, 2000):

- Type C (Chronic): These reactions are accompanied with using a particular drug for a long duration e.g. prednisolone treatment causes iatrogenic Cushing's syndrome as a result of elevated glucocorticoid levels.
- Type D (Delayed): These types of reactions can be observed years after the end of treatment. Secondary tumours resulting from alkylating agents is an example of a delayed reaction. This definition is also applicable to teratogenic compounds resulting in adverse effects to the embryo of mothers who are being treated while pregnant e.g. birth defects can be seen with valproate and thalidomide treatment.
- Type E (End of use): Take place upon drug withdrawal. They can be avoided in some instances by a gradual dose reduction. For example, sudden anticonvulsant withdrawal may cause seizures whereas β -adrenoreceptor antagonists' withdrawal is accompanied with unstable angina.

Unsafe drugs are still going on to the market despite rigorous *in vitro* and *in vivo* strategies of testing. A better understanding of underlying mechanisms is thus needed to develop strategies for better-quality screening of new chemical compounds.

1.3 Immunological reactions

The immune system is involved in type B (off-target) reactions. Adverse drug reactions caused by an immune response have a tendency to be severe in nature and are very difficult to predict as there are limited preclinical screens able to recapitulate the immune system complexity either *in vitro* or *in vivo*. These types of reactions will be the main focus of this thesis.

1.3.1 Classification of immunological reactions

Drug hypersensitivity reactions can be defined as a severe adverse drug reaction which is caused by an immune response despite of therapeutic agent effectiveness and safety. In 1963, Gell and Coombs were the first to group immunological reactions in to four categories based on the type and main clinical characteristics of the immune response. Antibody mediated reactions are represented as type I-III reactions, while reactions caused by T-cells are represented as type IV reactions. Gell and Coombs classification is still commonly in use today, with the additional subdivision of type IV reactions presented lately (Pichler, 2003). Table 1.2 summarised the main characteristics of each reaction type. However, it must be noticed that overlap between reaction features is an often seen in clinical practice, especially with regard to the release of specific mediators. Type IV (delayed type T-cell mediated) reactions will be discussed in more detail later.

Extended Coombs and Gell classification	Type of immune response	Pathologic characteristics	Clinical symptoms	Drug binding	Cell type
Type I	IgE	Mast cell degranulation	Urticaria and anaphylaxis	Covalent	B-cell
Type II	IgG and FcR	FcR dependant cell destruction	Blood cell dyscrasia	Covalent	B-cell
Type III	IgG and complement or FcR	Immunocomplex deposition	Vasculitis	Covalent	B-cell
Type IVa	Th1 (IFN- γ)	Monocyte activation	Eczema	Covalent/non-covalent	T-cells
Type IVb	Th2 (IL-5 and IL-4)	Eosinophilic inflammation	MPE, BE	Covalent/non-covalent	T-cells
Type IVc	CTL (perforin and granzyme B)	CD4 ⁺ or CD8 ⁺ cytotoxicity	MPE, eczema, BE and PE	Covalent/non-covalent	T-cells
Type IVd	T-cells (IL-8)	Neutrophil recruitment and activation	PE	Covalent/non-covalent	T-cells

Table 1.2- Extended Coombs and Gell classification of immune-mediated adverse drug reactions. MPE (Maculopapular exanthema), BE (Bullous exanthema), PE (Pustular exanthema). Adapted from (Pichler, 2003).

1.4 Immune activation

1.4.1 Components of the immune system

The immune response includes both innate and adaptive immunity. Innate immunity is a generalised, non-specific reaction usually resulting from infection or tissue trauma/damage. Adaptive immunity is an acquired reaction, which in particular recognises antigens. An enormous range of cell types are included in both innate and adaptive immune responses. Most but not all of the immune cells are continuously replaced as they have short life span. Pluripotent stem cells located in the bone marrow are the main origin of all cell types, which depend on different stimuli, involving hormones and cytokines for differentiation. Immune cells are grouped into categories, each with a particular task.

Lymphocytes

Leukocytes or white blood cells are immune cells involved in defending the body against foreign materials and infectious disease. Approximately 20% of the leukocytes found in blood are lymphocytes. Lymphoid progenitor cells produce natural killer (NK), B and T cell subsets and each one of them have their unique developmental pathways and functions. Initially B-cells develop in the foetal liver then they migrate to the bone marrow for further maturation. Production and secretion of antibodies is the main function of B-cells. Immunoglobulins are expressed by B-cells and they serve as antigen receptors. B-cells proliferate and differentiate into either memory or plasma cells following antigen exposure. The antibodies secreted by plasma cells exhibit the same antigenic specificity as the immunoglobulins expressed on the cell surface. These antibodies have a number of functions including complement fixation, the shielding or neutralisation of viral components and coating or opsonisation of antigen to enhance phagocytosis. Long-lasting memory cells are also produced but in a small number. They circulate in an inactive state but promptly respond when exposed to the antigen again. This can be related to the high affinity IgA or IgG molecules expressed on memory cells, whereas naïve cells express lower affinity IgD and IgM molecules. B-cells can be recognised experimentally by the expression of cell surface markers (e.g. CD19).

Immediate hypersensitivity reactions to drugs such as the β -lactam antibiotics are associated by the release of specific IgE antibodies (Torres et al., 2003).

T-cells originate from the thymus. Antigenic specificity of T-cell is determined by T-cell receptors and each T-cell will express only one TCR since they recognise only a small number of antigens. The TCR, in comparison to B-cells, recognises only a small part of the antigen with just a limited number of amino acids. When exposed to an antigen, antigen-specific T-cells bearing the corresponding TCR will divide and differentiate to produce an abundant population of effector T-cells. A great number (>95%) of newly developed thymocytes will be removed by positive selection (Romagnani, 2006). Some T-cells will die as a result of negligence as their expressed TCRs have no affinity for MHC-peptide complexes. After that surviving cells go through negative selection which stimulates apoptosis only in T-lymphocytes expressing TCRs with high affinity for self-peptides that exist on the medullary epithelial cell surface (Romagnani, 2006). T-cells subsets express either CD8 or CD4 molecules. Initially this was thought to relate to the function of effector T-cells; CD8⁺ T-cells were denoted as cytotoxic cells while CD4⁺ T-cells were helper cells. However, this peculiarity is no longer 100% applicable as some CD4⁺ T-cells can also exhibit cytotoxic activity (Marshall and Swain, 2011).

Effector cytotoxic CD8 T cells (CTLs) provide important protection against a range of intracellular viral and bacterial infections and are in charge for anti-tumour responses (Barber et al., 2006; Bos et al., 2012; Condotta et al., 2012). Infected cells exhibit pathogenic peptides derived from cytosolic proteins which can be recognised by CD8 T cells, as MHC class I proteins present on the surface of almost each nucleated cell of the body. The significance of CTL function is highlighted in different settings of diseases such as chronic viral infections involving human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV) and tumours involving melanoma, prostate, lung and ovarian malignancies which are an outcome of CTL dysfunction (Shin and Wherry, 2007; Matsuzaki et al., 2010; Chou et al., 2012; Fourcade et al., 2012; Prado-Garcia et al., 2012). Activated antigen-specific CD8 T cells start to secrete IL-2 upon dendritic cell mediated

competent co-stimulation and antigen stimulation in the lymph node. This functions in an autocrine mode by binding to its high affinity IL-2R on the surface of T cell, therefore driving its functional differentiation and exponential clonal expansion into effector CTLs (Boyman and Sprent, 2012). CD8 T cell differentiation is mainly dependent on the transcriptional activity of T-box transcription factors, T box expressed in eomesodermin (EOMES) and T cells (T-bet) (Kaeck and Cui, 2012). Then the resultant CTLs enter into the blood stream and migrate to infection sites where their effector activity generally uttermost at day seven post-infection (Obar et al., 2008). Cytotoxic T lymphocytes attack infected cells via various mechanisms including cytokine secretion and cellular-mediated cytotoxicity (Topham et al., 1997; La Gruta et al., 2004). CTLs secrete cytolytic effector molecules including the pore-forming perforin and proteases granzyme A and B upon antigen recognition in combination with MHC I on the target cell. Perturbation of the target cell membrane by perforin enhances the entry of granzyme A and B which starts a caspase apoptotic dependent pathway, eventually leading to cell death (Lieberman, 2003). In addition, CTLs secrete proinflammatory cytokines such as TNF- α and INF- γ (La Gruta et al., 2004). Inhibition of viral replication and promotion of MHC I expression on infected cells are enhanced by INF- γ . This result in increasing infected cells probability of being targeted by activated CTLs. Secretion of TNF- α stimulates innate immune cells and activates caspase-dependent cellular apoptosis through ligation with its TNFR-1 cognate receptor on the target cell. Co-stimulation is vital for CTL effector role. Obviously, CD28 signalling is accompanied with stimulation of IL-2 secretion. Subsequently, this initiates functional differentiation of CTLs via increasing perforin and granzyme expression (Janas et al., 2005; Williams et al., 2006).

One of the important factors that affect the CTL effector response is the CD8 TCR antigen affinity. In general, cross-presented tumour or self-antigens with low avidity will be disregarded by CD8 T cells, leading to absence in effector response even in the existence of a viral infection (Bos and Sherman, 2010). CD8 T cells with a higher affinity for the tumour antigens, leads to enhanced granzyme B and perforin expression, induction of tumour irradiation and decline in co-inhibitory receptor expression (Bos et al., 2012). In these situations the requirement for greater CTL effector function is enabled by the provision of CD4 T cell help (Bos and Sherman,

2010). CD8 T cell stimulation and differentiation is enhanced by CD4 T cells through different mechanisms including secretion of IL-2 and IFN- γ and activated CD4 T cell (Bos and Sherman, 2010). CD4 T cells can also directly interact with dendritic cells through ligation of CD40:CD40L. This allows upregulation of dendritic cell CD80/86 co-stimulatory receptor expression, leading to more proficient priming of CTL immune response (Bos and Sherman, 2010).

CD4⁺ T-cells can be divided based on the cytokines they secrete. Naïve cells are named as type 0 (Th0) cells, prior to antigenic exposure. When IL-2 is present, Th0 cells are activated and convert into Th1 cells. In contrast, in the presence of IL-4, Th2 cells will be produced. Subsequently Th1 cells secrete IL-2 and IFN- γ , while Th2 cells secrete IL-13, IL-4, IL-5 and IL-9 when activated (Mosmann and Coffman, 1989). These various cytokine profiles then affect the immune response that takes place; Th1 cytokines induce cell-mediated immunity while a humoral (B-cell) response will be induced in the presence of Th2 cytokines. One of the relatively newly discovered T-cell subset is the Th17 secreting cells (Harrington et al., 2005). These cells are believed to play a role in some diseases such as multiple sclerosis and rheumatoid arthritis (Tesmer et al., 2008). It is believed that IL-17 secreting T-cells can develop along this lineage following a signal from IL-1 β (Acosta-Rodriguez et al., 2007). Drug-specific T-cells with differing cytokine profiles have been isolated from the peripheral blood of patients with different forms of drug-induced skin reaction. In contrast, the role of T-cells in drug-induced liver injury is less well-defined.

A small number of lymphocytes are classified as natural killer (NK) cells based on expression of the cell surface marker CD56. They respond promptly to viral infection as they do not express antigen receptors. NK killing activity is mediated by granzyme and perforin molecules which are released either directly or in vesicles. It was thought previously that NK cell activation is non-specific; however recent studies show activation is dependent upon the expression of a number of activating and inhibitory receptors. Killer-cell immunoglobulin-like receptors (KIRs) are an example of one of the inhibitory receptors and they are specific for a variety of

ligands including MHCclass I molecules. CD244, DAP-12 and NKG2D are all considered as activating receptors (Lanier, 2008). The balance between the activities of these receptors is the main determinant of the activation state of the NK cell.

Phagocytes

The main role of the phagocyte is to engulf and digest pathogens such as bacteria. This can be attained in different ways and the various phagocyte subsets are discussed below.

The myeloid progenitor cell is the origin of monocytes. Monocytes migrate into tissues after circulating in blood. They then differentiate into macrophages. Macrophages have the ability to break down pathogens as they have numerous lysosomes containing digestive enzymes. Cell surface expression of CD14 is used as a marker to identify these cells *in vitro*. Generally, macrophages remain in tissues when matured and only a small number will return to the circulation. Highly-specialised macrophages that reside in liver are known as kupffer cells.

Approximately 70% of the leukocytes found in the blood are neutrophils. Chemoattractants such as IL-8 influence the migration of neutrophils into the tissue as they are somewhat short lived in the circulation. Neutrophils contain several different granule types hence they are often referred to as granulocytes. These include acidic lysosomes and tertiary, secondary and primary granules. They contain digestive molecules such as myeloperoxidase and lactoferrin. Neutrophils are recruited to sites of local tissue damage and activation results in degranulation and consequently recruitment of other cell types.

Accessory cells

Accessory cells work along with lymphocytes to induce an immunological response. Mast cells, basophils and eosinophils cells all have a granular morphology and secrete different mediators when stimulated. Major basic protein is the main constituent of eosinophil granules and it is a cationic molecule able to directly destroy pathogens. Eotaxin and IL-5 secretion results in eosinophil recruitment to an inflammation site. The main mediators of the inflammatory response i.e. prostaglandins, cytokines, leukotrienes are synthesised by eosinophil. Basophil granules comprise inflammatory mediators such as leukotrienes, IL-4 and histamine. The main constituent of mast cell granules is also histamine.

Dendritic cells (DCs) are often referred to as professional antigen presenting cells. Circulating DCs that have low levels of costimulatory molecule and MHC class II expression and low T-cell activation potential, but high endocytic activity are referred to as immature cells and they develop into mature DCs upon antigenic exposure. Maturation leads to an increase in costimulatory molecule expression (Sallusto et al., 1998), decreased endocytic capability (Cella et al., 1996), but increased levels of MHC class II expression (Sallusto et al., 1995). Other signals that enhance maturation of dendritic cells include toll-like receptor signalling by components of bacteria and secretion of damage-associated molecular patterns (DAMPs) such as heat shock proteins, HMGB1 and uric acid, and release of pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) (Shi et al., 2003).

1.4.2 Antigen processing, presentation and cross presentation

The key element of adaptive immunity is specific-antigen recognition by T-cells. As mentioned previously, the TCRs expressed by T-cells are specific for MHC-peptide complexes. Appropriately, most antigens must be presented in the context of an MHC molecule to be recognised.

Antigen-presenting cells follow two distinct pathways for antigen processing [Figure 1.1]. The first pathway involves intracellular antigens bound to MHC class I molecules. Viral infection is one of the usual forms of intracellular antigen. Viral proteins are secreted into the cell cytosol of the host following virus entrance and replication. Proteosomes degrade the proteins either in the nucleus or cytosol. Subsequently, peptides are translocated to the endoplasmic reticulum (ER) through a transporter accompanied by antigen processing (TAP)-dependent pathway. The MHC class I heterodimer is then formed in the ER with the peptide fragment needed for stability (Neefjes et al., 2011). For antigen presentation, the complex is released and travels to the surface of the cell. MHC molecules not associated with peptides are returned to the cytosol to be degraded (Hughes et al., 1997). Intracellular derived peptides are presented to CD8⁺ T-cells by MHC class I molecules.

The second pathway involves extracellular antigens. Highly specialised antigen-presenting cells such as macrophages and dendritic cells are the only cells which can process antigens in this particular way. Pathogens enter the cell by phagocytosis and enclosed into an endosomal vesicle where they are digested by enzymes into peptide fragments. MHC class II molecules are transported in membrane-enclosed vesicles from the endoplasmic reticulum. These vesicles then fuse with the endosome permitting peptide loading to occur. MHC class II molecules are then transported to the plasma membrane in order to present their loaded peptide fragments to CD4⁺ T-cells (Neefjes et al., 2011).

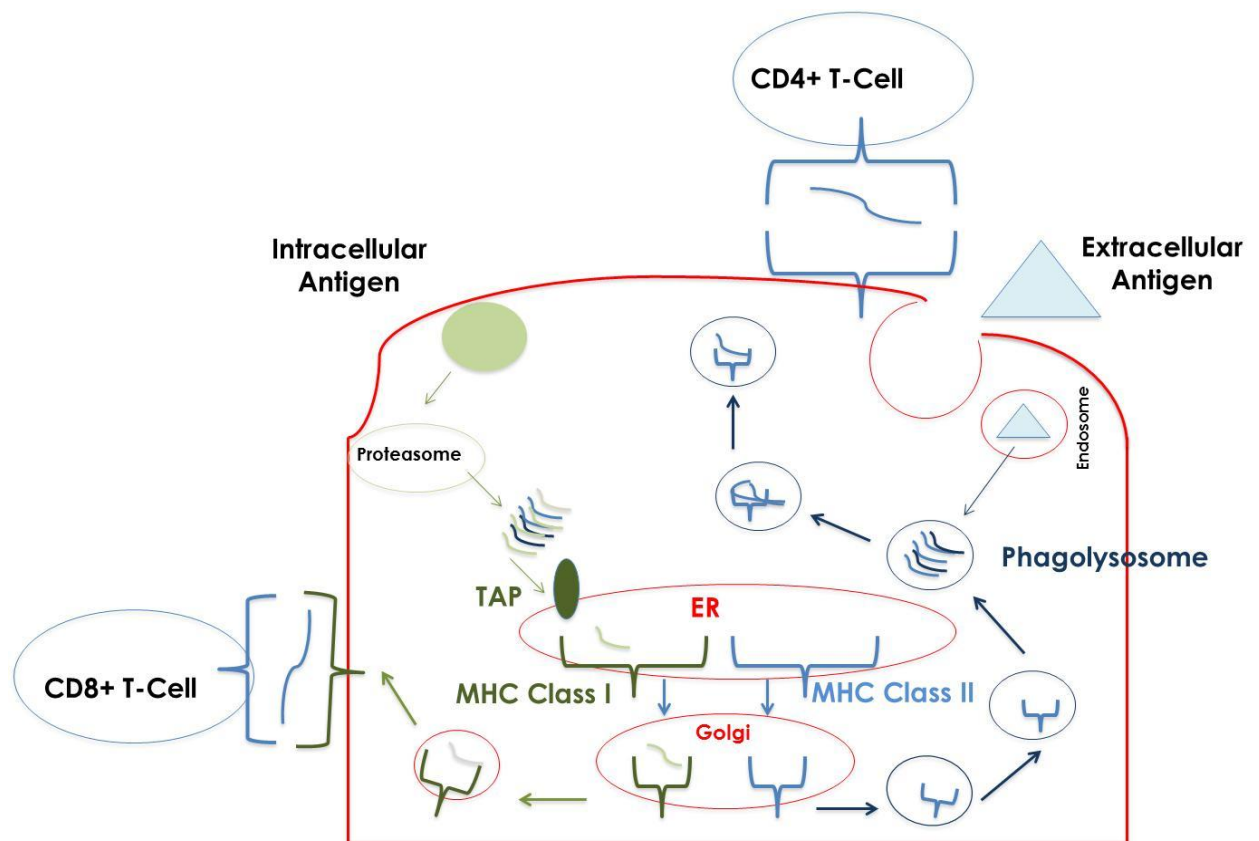


Figure 1.1- Antigen processing and presentation. Extra and intra-cellular pathways of processing. Extracellular antigens engulf by phagocytosis and degraded by endosomal enzymes. MHC class II molecules are transported in vesicles coming from the endoplasmic reticulum (ER) and golgi apparatus. These vesicles fuse together with the phagolysosome and this is where loading of peptide occurs. The peptide bound MHC is then transported to the surface of the cell where the antigen is displayed. Intracellular antigens are broken down in the proteasome. Peptides are transported to the ER through TAP and attach to MHC class I molecules. The peptide bound MHC is then transported again to the golgi where it is loaded into a vesicle and trafficked to the surface of the cell. Adapted from (Neefjes et al., 2011).

Some dendritic cell subsets particularly the CD8⁺ lymphoid dendritic cells can uniquely present exogenous cell associated antigens from infected or dying cells in the context of MHC I molecules to naïve CD8 T cells, a phenomenon called “cross-presentation” (den Haan et al., 2000; Belz et al., 2005). In the case of intracellular viral infection, presentation of MHC I peptide complex to CD8 T cells depends on the dendritic cell to be infected itself as shown with the classical endogenous MHC I pathway. However, viruses can make use of certain mechanisms to severely encumber MHC I presentation in infected dendritic cells therefore avoiding cytotoxic CD8 T cell immunity (Nopora et al., 2012). Mechanisms of cross priming provides a

way by which uninfected dendritic cells can stimulate CD8 T cell immune responses via the processing of cell-associated antigens derived from endogenous viral infected cells (Nopora et al., 2012). Moreover, it is important for priming CD8 T cell anti-tumour reactions in response to exogenous tumour self-antigens expressed by unhealthy or dying cells (Hoffmann et al., 2000). Not like, the exogenous MHC II pathway, cross-presentation takes place in early endosomes or nascent phagosomes (Houde et al., 2003) typified by their neutral pH and mostly depends on their exogenous material transport into the cytosol for proteasomal degradation (Blum et al., 2013). Thus, maturation of phagosomes into advanced proteolytic active endosome compartments is not essential. Similar to MHC I presentation, cross-presentation is a constitutive process in immature dendritic cells and has a significant role in maintaining CD8 T cell tolerance (Kurts et al., 1998). This proceeds via immature dendritic cell presentation of extracellular self-antigens in the context of MHC I to autoreactive CD8 T cells, which leads to their deletion from the T cell repertoire and eventually prevents the beginning of autoimmune responses (Kurts et al., 1997; Kurts et al., 1998; Heath and Carbone, 2001). For instance the cross-presentation of cell-associated antigens derived from necrotic or apoptotic cells. These cells are internalised via phagocytic mechanisms into phagosomes and are incompletely degraded, after that they are expelled to the cytosol and headed for proteasomal degradation (Blum et al., 2013). The consequential antigenic peptides are then transported to the endoplasmic reticulum through TAP where they are loaded onto MHC I molecules and transported to the cell surface (Kovacsovic-Bankowski and Rock, 1995). The phagosome itself is also able to obtain the optimum components for efficient exogenous MHC I peptide loading through fusion with endoplasmic reticulum derived vesicles. In this respect, the acquired dying cell is to some extent degraded in the endoplasmic reticulum fused phagosome and the yielded proteins are transported from the fused phagosome to the cytosol through the transporter Sec61, where they are directed to the proteasome for more degradation. The yielded peptides are then returned back into the phagosome through TAP where the peptide is loaded onto the MHC I and transported to the dendritic cell surface (Houde et al., 2003). Cross-presentation can also carry on through proteasome independent mechanism and TAP (Bachmann et al., 1995). In this mechanism, acquired dying cells are transported through the phagosome to vacuolar post-golgi compartments where they are degraded into smaller peptide fragments through

proteases. Recycled MHC I from the dendritic cell surface is attained within these phagocytic vesicles. The peptide is then loaded onto the MHC I and the MHC I peptide complex is transported afterward to the dendritic cell surface (Trombetta and Mellman, 2005).

1.4.3 Intracellular signalling following activation of TCR

A signalling cascade is triggered when the MHC-peptide is engaged with the TCR. This causes cellular differentiation, proliferation and/or growth factor/cytokine release. The process of the signal transfer across the cell membrane (TCR-triggering) is complex, and not fully understood. Currently suggested models propose either conformational segregation, aggregation or change of the TCR-CD3 complex (Choudhuri et al., 2005). Much more is understood about the signalling cascades after TCR triggering. An early event in T-cell signalling is stimulation of tyrosine protein phosphorylation by the Src kinases Fyn and Lck (Nel, 2002). Immunoreceptor tyrosine phosphorylation based activation motifs (ITAMs) within CD3 by itself functions as a docking site for other proteins particularly ZAP-70 (Smith-Garvin et al., 2009). In this manner the TCR comes to be a phosphorylation site for an enormous range of substrates commencing multiple signalling pathways that eventually result in T-cell activation. ZAP-70 targets are the SH2 domain-containing leukocyte phospho-protein of 76kDa (SLP-76) and a transmembrane adapter protein linker for T-cells (LAT). The eventual consequences of TCR activation involve activation of enzymes (e.g. PKC, ERK, JNK), release of transcription factors (e.g. STAT3, NFAT) and the production of signalling molecules such as DAG and Ca^{2+} .

1.5 Drug-induced skin injury (DISI)

In T-cell mediated hypersensitivity reactions, the skin is the organ most frequently involved; either in isolation or as a component of a generalised hypersensitivity reaction. The reason why skin is affected much more commonly than other organs may be due to the rich vascularisation, large surface area, dense network of T-cells and APCs ready to stimulate an immunological response (Keller et al., 2005; Clark et al., 2006). The severity of skin injury symptoms depend on both the individual and the chemical/drug administered. Reaction severity ranges from mild inflammation to life-threatening reactions such as Stevens-Johnson syndrome.

The term “drug induced skin injury” was proposed by Pirmohamed and his colleagues in the Phenotype Standardisation Project (PSP). The main objective of this consortium was to improve the phenotyping of various types of severe adverse drug reaction (Pirmohamed et al., 2011b). Mainly three types of adverse drug reactions were included under the umbrella of this project: drug induced skin injury (DISI), drug induced liver injury (DILI) and drug induced torsade de pointes (DITdP). The main goal for the PSP was to address the necessity for sufficient cohorts of patients to study the genomic basis of certain adverse drug reactions to determine individuals at risk. The project also aimed to ensure correct epidemiological patient classification to enable regulators, industry researchers and clinicians to correctly identify various types of adverse drug reactions (Pirmohamed et al., 2011a). The focus in the following discussion will be mainly on the serious types of adverse drug reactions involved in both DISI and DILI.

Drugs can stimulate various forms of DISI including maculopapular exanthema (MPE), acute generalised exanthematous pustulosis (AGEP), Steven-Johnson Syndrome (SJS), toxic epidermal necrolysis (TEN) and drug reaction/rash with eosinophilia and systemic symptoms (DRESS). The immune pathophysiology and clinical features of these serious reactions is discussed below.

1.5.1 Immune cells in the skin

Traditionally, it was thought that the skin is no more than a biochemical and physical barrier defending organisms from external agents. However, in recent years it has been revealed to have an important role in the immunological response, as its cellular components includes macrophages, mast cells, keratinocytes, dermal dendritic cells and epidermal Langerhans cells, which function as static skin constituents to release proinflammatory cytokines and enhance the recruitment of other cells that are part of the dynamic immune response. These cells include antigen-presenting cells (APCs) such as macrophages, monocytes, dendritic cells and T lymphocytes expressing skin-homing receptors such as the cutaneous lymphocyte antigen (CLA) and chemokine receptors (e.g. CCR6, CCR4, CCR10), which represent the cellular baseline of the immune memory within the skin (Bos and Kapsenberg, 1993; Blanca et al., 2000; Fernandez et al., 2009).

Keratinocytes are located in the epidermis and plays a key role in DISI. Keratinocytes detect pathogens by expressing several pattern recognition receptors, including Toll-like receptors (TLRs) that identify a wide range of pathogen components, Nod-like receptors (NLRs) Nod1 and Nod2, which react to bacterial peptidoglycan and NLR pyrin domain-comprising proteins that interact with fungal and viral pathogens. C-type lectins such as dectin-1 respond to fungal infections, whereas expression of RIG-I-like receptors (RLRs) supports detection of viral RNA (Heath and Carbone, 2013).

Keratinocytes can release immense range of microbiocides including antimicrobial peptides such as β -defensins and LL-37, as well as S100 and RNases family members. Chemokines and cytokines can also be produced by keratinocytes in response to pathogen invasion, including CCL20, CXCL11, CXCL10, CXCL9, IL-33, IL-18, IL-10, IL-6, IL-1 α , IL-1 β and TNF- α (Nestle et al., 2009; Meephansan et al., 2012). Cytokines support effectors and direct the immune response to guarantee effective elimination of pathogens, whereas chemokines are crucial regulators of effector cell migration in the skin (Zhu et al., 2010; Nagao et al., 2012).

Dendritic cell (DC) subsets existed in the human skin have been studied extensively. There are four different subsets of DCs in the human skin including Langerhans cells, CD14⁺ DCs, CD1c⁺ DCs and a recently identified CD141⁺ DC subset (Haniffa et al., 2012). The CD141⁺ DC subset is important for viral immunity and CD8⁺ T-cell responses. The other important DC subset is the Langerhans cells, which are located in the epidermis above the keratinocytes. Traditionally, Langerhans cells were thought to be responsible for priming skin immunity. Furthermore, researchers have shown they have an important role in the initiation of immune responses against contact allergens (Allan et al., 2003; Kaplan et al., 2005). It has also been shown that Langerhans cells play a role to priming skin immunity against pathogens such as bacteria (e.g. *Staphylococcus aureus*) and yeast (e.g. *Candida albicans*) through differential stimulation of Th17, Treg and effector T-cell (Heath and Carbone, 2013).

A number of $\alpha\beta$ T-cells reside in the skin may have a memory phenotype. These memory T-cells are CD4⁺ and CD8⁺ positive, with approximately 10% of the CD4⁺ T-cells expressing Foxp3 representing a Treg phenotype (Heath and Carbone, 2013). A subset of cells is called tissue-resident memory cells (T_{RM}); these cells are disconnected from the blood circulation and remain permanently in the skin. These cells are thought to provide protection against a range of pathogens (Gebhardt et al., 2009).

1.5.2 Clinical presentation and mechanisms of DISI

The reasons why a drug administered parentally or orally commonly targets the skin have not be fully defined. Most of DISI (>90%) is benign MPE (Hunziker et al., 1997). Reactions initially appear on the upper extremities and trunk then spread more widely (Roujeau, 2005). Usually reactions are detected in the first two weeks after drug treatment; however, reactions with a later onset have been reported.

All types of DISI differ in the effector cell phenotype and cytokine secretion profile of activated T-cells. CD8⁺ T-cells are thought to be dominant in TEN, whereas CD4⁺ T-cells dominate in MPE and AGEP. Research studies on skin have revealed that IL-5 is the main cytokine in MPE. IL-5 is important as it is involved in the recruitment of eosinophils to the site of inflammation (Pichler et al., 1997). IFN- γ and IL-8 are released by drug-specific T-cell in patients with AGEP (Britschgi et al., 2001; Roujeau, 2006). IFN- γ upregulates MHC class II on keratinocytes surface making them more prone to T-cell mediated killing, while IL-8 is involved in the recruitment of neutrophils to the site of inflammation (Friedmann et al., 1994; Roujeau, 2006). Patients with SJS/TEN have high levels of granulysin in their blister fluids (Nassif et al., 2004b; Chung et al., 2008), together IFN- γ and TNF- α , which collectively induce keratinocyte cytotoxicity (Friedmann et al., 1994).

DRESS is also known as hypersensitivity syndrome (HSS). DRESS begins later than other types of DISI and can be seen even after 2-6 weeks after treatment initiation. Liver involvement can increase the mortality rate of DRESS up to 10% (Roujeau, 2005). Drug-specific T-cells that release high levels of IFN- γ and IL-5 are detected in the blood of patients with DRESS (Naisbitt et al., 2003b). Viral reactivation is frequently observed in the course of DRESS; thus, it can be utilised as a diagnostic criterion (Suzuki et al., 1998; Tohyama et al., 1998). Still, it is not fully understood what role this reactivation has in the pathophysiology of the disease and whether it is a consequence or cause of the clinical manifestations. Reactivation of human herpes virus 6 (HHV-6) is detected by increasing levels of particular viral DNA and IgG. However, this usually is not measurable until many weeks after symptoms appear (Shiohara et al., 2006). Thus, it is likely that viral reactivation is caused by T-cell activation. Transient increases in virus-specific immunoglobulins against HHV-7, Epstein-Barr virus (EBV) and cytomegalovirus (CMV) have also been detected in patients with DRESS (Aihara et al., 2001; Descamps et al., 2003; Picard et al., 2010). Cells from skin lesions have been analysed and found to contain high levels of HHV-6 DNA and express viral antigen at an early stage (Suzuki et al., 1998). Therefore it is still theoretically possible that certain clinical manifestations result from virus-specific T-cells expansion (Shiohara et al., 2006).

SJS/TEN is the most serious form of DISI. The main characteristic is blistering skin lesions. Nowadays, it is believed that both SJS and TEN are involved within the same disease spectrum with different degree of skin detachment and this difference is being used to delineate each type (Bastuji-Garin et al., 1993; Mockenhaupt, 2011). SJS and TEN have a high mortality rate with approximately 10% and >30%, deaths reported respectively. Death usually happens as a consequence of pulmonary symptoms or sepsis. Treatment often requires a specialist from burns unit with expertise in dealing with extensive skin loss. Table 1.3 illustrates the common characteristics of SJS/TEN and DRESS.

TEN is a CD8+ T-cell mediated reaction (Schwartz et al., 2013). Histopathology shows that keratinocyte death in the epidermis initially results from apoptosis, which subsequently leads to necrosis and skin detachment (Paul et al., 1996). NK T-cells are also involved in the pathogenesis as the skin lesions contain high numbers of these cells (Chung et al., 2008). Blister fluid contains high levels of the soluble mediator granulysin and granulysin levels have been shown to correlate with clinical severity (Chung et al., 2008). Therefore, measurement of granulysin levels in the serum of patients with TEN can be used as an early diagnostic marker (Abe et al., 2009).

Symptom	Drug-induced skin injuries		
	SJS	TEN	DRESS
Mucous membrane	>90%	>90%	<30%
Erosions	Several sites	Several sites	Mouth and lips
Detachment of epidermis	Yes <10% of BSA	Yes >30% of BSA	No
Hyperkeratosis/desquamation	No	No	Usual
Neutropenia	NO	30%	No
Eosinophilia	No	No	90%
Atypical lymphocytes	No	No	30-40%
Respiratory tract	Bronchial Erosions/ARDS	Bronchial Erosions/ARDS	Interstitial pneumonitis
Liver	Hepatitis 10%	Hepatitis 10%	Hepatitis 60%
Heart	No	No	Myocarditis
Lymph node enlarged	No	No	Usual
Viral reactivation	Occasional	Occasional	Frequent

Table 1.3- Features of the most serious DISI. (BSA) body surface area, (ARDS) acute respiratory distress syndrome. Adapted from (Bachot and Roujeau, 2001).

1.6 Drug-induced liver injury (DILI)

Drug-induced liver injury is a rare but serious health problem with an incidence rate of 1 in 10,000 and 1 in 100,000 (Holt and Ju, 2006). DILI is one of the main causes for the withdrawal of newly marketed drugs. Identifying hepatotoxic drugs during the development process is not easy, because reactions are not predictable in pre-clinical animal models or in *in vitro* humanised experimental systems (Kaplowitz, 2005). This is particularly applicable with the idiosyncratic reactions that are thought to involve the immune system (Holt and Ju, 2010). DILI is usually seen when the drug is marketed and exposed to several thousand patients (Watkins, 2005). Cautious monitoring is needed, because liver injury is often not seen until weeks or even months after the initiation of drug treatment (Wilke et al., 2007).

1.6.1 Immune cells in the liver

The liver is an organ that is rich in haematologic and lymphoid cells. A vast array of immune cells which participate in both innate and adaptive immunological responses are present. The innate cells in the liver include kupffer cells (liver resident macrophages), NK cells, NK T-cells and liver sinusoidal endothelial cells. They come in to contact with the antigens circulating in the blood passing via the liver sinusoids and act as a first line protection. For the liver, it is vital to maintain a tolerance state to a wide range of antigens, as it is constantly exposed to antigens from the gastro-intestinal tract. This is accomplished by the host genetics and by maintaining high stimulus threshold before activation (Knolle and Gerken, 2000). Kupffer cells and sinusoidal endothelial cells are able to present antigens to T-cells, which may activate their effector function. The subsequent response is primarily based on the local production of chemokine and cytokine, since they control the recruitment of immune cells to the site of inflammation and/ or infection (Knolle and Gerken, 2000).

1.6.2 Mechanisms of DILI

Liver cell injury occurs in different patterns based on the affected organelles. Cell membrane injury can lead to disturbance of calcium homeostasis: Mg^{+2} and Ca^{2+} ATPases are essential molecules that sustain the intracellular balance. Reactive drug metabolites can attach to important proteins resulting in loss of cellular integrity disturbance. Disturbed level of intracellular calcium leads to actin filament disassembly, cytoskeleton dispersal, bleeding of the cell membrane, and subsequently rupture and lysis of the cell (Watanabe and Phillips, 1986; Cullen, 2005).

Cholestatic and canalicular injury: to enable bile salt secretion a series of ATP-dependent transporters are required. These transporters enhance bile movement from hepatocytes to the canalicular lumen. Chemicals that attach to canalicular transporter molecules can induce cholestasis leading to the prevention of bile formation or circulation within the lumen (Lee, 2003; Cullen, 2005).

Metabolic bioactivation: Cytochrome P450 is a group of oxidising enzymes found in hepatocytes, within the smooth endoplasmic reticulum. They are responsible for phase-I drug oxidation, converting the parent compound into more hydrophilic intermediates that undergo phase-II conjugation. The most common mechanism of hepatocellular injury includes secretion of harmful reactive metabolites by the cytochrome P450 system. Conjugation by phase-II enzymes generally controls exposure to reactive intermediates and prevents toxicity. However, reactive metabolites with high energy may exceed the detoxification pathway and produce an adduct with cellular proteins. Adduct formation can inhibit essential cellular processes, resulting in stress and cellular injury through apoptotic or necrotic mechanisms (Baron and Merk, 2001; Oesch et al., 2007).

Drugs can theoretically stimulate the hepatic adaptive immune system via either a prohaptent/haptent mechanism or pharmacological interaction (p-i) pathway. To date,

there is only one study characterising drug-specific T-cell responses in patients with DILI. Maria and Victorino detected drug-specific proliferative responses in approximately 50% of patients with DILI using the lymphocyte transformation test (Maria and Victorino, 1997). However, these lymphocytes were not characterised in terms of phenotype and function and additional studies have not been forthcoming.

1.6.3 Clinical presentation of DILI

Clinical presentation of DILI can take the form of any of the following:

- Acute hepatitis which presents as nausea, fever, malaise, jaundice (in certain patients) and a raise in serum transaminases. Pathohistological examination reveals necrosis of liver parenchyma and infiltration of the portal tract with immune cells particularly eosinophils, plasma cells and lymphocytes. Viral hepatitis shows a similar pathohistological picture. Drugs such as carbamazepine, rifampicin and isoniazid are associated with this particular type of liver injury (Talbot, 1975).
- Cholestatic hepatitis presents commonly with jaundice, high levels of conjugated bilirubin and serum transaminases. Pathohistological examination reveals retention of bile in bile canaliculi and hepatocytes. Other features of this type of DILI include infiltration of lymphocytes in the portal tract and minimal hepatocyte necrosis. The most common causative therapeutic drugs include methyldopa, chlorpromazine, dapsone, cotrimoxazole (Talbot, 1975) and flucloxacillin (Russmann et al., 2005).
- One of the most severe forms of DILI is hepatic necrosis with confluent necrotic patches in liver parenchyma; hepatocytes are usually surrounded by fat infiltration. Patients pass in to coma following a period of restlessness. Eventually coagulopathy of blood and jaundice are apparent. Drugs associated with this fulminant type of liver injury e.g. monoamine oxidase inhibitors, halothane and imipramine (Talbot, 1975).

1.7 The major histocompatibility complex

The key aspect of drug hypersensitivity research is individual variation. Susceptibility of certain individuals to a reaction is influenced by polymorphisms in drug metabolising enzymes. Recent genome-wide association studies however propose a much more important role for genes within the region of MHC.

1.7.1 Location and variation

The MHC region consists of a cluster of genes around 3.4Mb on the short arm of chromosome 6. Genes in this particular region play an important role in both innate and adaptive immunity (Daly et al., 2001). Within the human genome, the MHC region is the most polymorphic region. The MHC class I and II genes in humans are specifically termed as human leukocyte antigen (HLA) class I and II genes.

1.7.2 MHC class I

Almost all nucleated cells express MHC class I molecules. They include the HLA-A, HLA-B and HLA-C loci. Class I molecules involve one gene encoding a polymorphic light chain (α) and share a common β -chain (β_{2m}). The latter gene is not situated in the MHC region. β_{2m} plays an important role in transporting newly synthesised MHC molecules to the cell surface. The peptide-binding groove can accommodate peptide of between lengths 9 and 11 amino acids and is constrained at both ends. Van der Waals forces and hydrogen bonds both help secure the peptide in place.

1.7.3 MHC class II

In comparison to the MHC class I molecules, class II molecules are only expressed by highly specialised antigen-presenting cells like B-cells, macrophages and dendritic cells. They include the HLA-DR, HLA-DQ and HLA-DP loci. Both genes for the α and β chains are situated in the MHC region. Unlike MHC class I

molecules, MHC class II molecules can accommodate longer peptides in their peptide-binding groove since they are open at both ends.

1.7.4 MHC class III

The MHC region is the site of many other genes besides the genes encoding antigen-presenting MHC class I and II molecules. These genes play a key role in the immunological response. They involve loci encoding genes linking to the complement cascade, inflammatory mediators such as leukotrienes, heat shock proteins such as Hsp70 and cytokines such as TNF- α .

1.7.5 HLA nomenclature

The array of HLA alleles that are now known were not anticipated when first discovered. Therefore several changes have been made to the way in which they are identified. HLA-A and HLA-B antigens were initially described by Dausset in 1958 (Dausset, 1958). The HLA-C locus was not identified until the year 1970 (Thorsby et al., 1970). And eight years later, the first class II alleles were discovered (Albert et al., 1978). Original classification relied on serological typing using the microlymphocytotoxicity assay of (Terasaki and McClelland, 1964). T and B-lymphocytes were collected from the patients and exposed to an array of antisera containing antibodies to each HLA molecule. Reactivity of the antibody was used to identify the antigens expressed at the cell surface. However, sequencing of the gene now means that individual amino acids variations that could not be identified using serologic techniques are now detectable. This required a new technique for naming alleles and it is this system that is still used today. The locus is identified by the letter immediately following HLA (e.g. A, B, C). The star immediately succeeding indicates that the allele has been allocated depending on molecular typing methods. Then a series of numbers follow this. The first two digit numbers indicate the type. In most cases this corresponds to the type that would be given by serology. The subtype is identified by the next two numbers. These numbers are assigned sequentially when new alleles are identified and highlight variations in the sequence of the amino acid. Both the fifth and sixth digits are used to signify polymorphisms that do not

influence the peptide sequence. Also digits number seven and eight can acknowledge polymorphisms in the non-coding region [Figure 1.2]. Nowadays, colons are utilised to separate each of these numbers. This classification allows 99 variations in each sub-type (i.e. variations are not any more constricted to two digits (Tait, 2011)). In order to provide information regarding allele expression, letters are included at the end of the numbered sequence, they include:

- N - null allele i.e. not expressed at the surface of the cell
- L - low expression at the membrane of the cell
- S - expressed in a form of soluble product
- C - expressed in the cytosol and not in the cell membrane
- A - aberrant expression
- Q - questionable expression, mutation can affect expression

To be able to discuss how to deal with the growing variety of HLA alleles, meetings are arranged on a regular basis. Next generation sequencing (NGS) signifies novel sequencing technologies that allow the generation of a high value of clonal sequences in a single sequencing run (Gabriel et al., 2014). Initially, next generation sequencing was introduced for quantitation of viral variants or genetic mutations in tumour tissues and for whole genome sequencing; more recently, the potential for HLA typing in a high resolution and high throughput analysis has been explored. It became very clear that the HLA system complexity allude to new challenges, particularly for bioinformatics. Realising the full potential of next generation sequencing will necessitate the development of particularly adapted software algorithms and typing strategies (Gabriel et al., 2014).

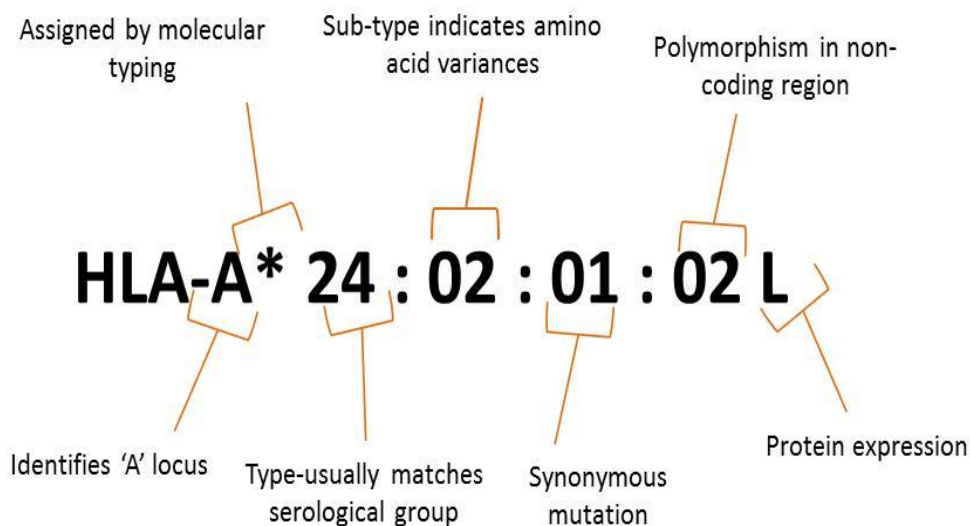


Figure 1.2 – Nomenclature of HLA allele.

1.7.6 Mechanisms of T-cell activation by drugs

The activation or stimulation of the immune system by small molecules such as drugs reveals an interesting dilemma knowing that the traditional target of the immune system is proteins or peptides. Different elements can determine drug antigenicity including: the environment i.e., viral or bacterial infection, drug i.e., drug chemistry and host i.e., metabolic system, disease and genetics. A number of various mechanisms by which a small molecule such as drug can interact with immune receptors have been postulated and are delineated below. These different mechanisms are not mutually exclusive.

1.7.6.1 Hapten hypothesis

Researchers in the 1930s were the first to provide an insight into the underlying mechanisms responsible for T-cell activation by small chemical allergens. Guinea pigs were sensitised by Landsteiner and Jacobs to chemically reactive low molecular weight compounds such as dinitrochlorobenzene (DNCB). The authors postulated that protein conjugation (adduct formation) may play an essential role in initiation of the observed immune response (Landsteiner and Jacobs, 1935). Low molecular weight chemicals are not likely to act as traditional antigens because of their small

size i.e., <1000 Da. Hence, the proposal that immune recognition only happens after a drug binds irreversibly to host proteins seemed logical. Landsteiner and Jacobs found a direct correlation between protein reactivity and immune sensitisation. This original observation resulted in the development of the hapten hypothesis. It is applicable to drugs such as the β -lactams antibiotics and to directly reactive protein chemicals. However, it should be noted that the origin of relevant protein modifications and the nature of antigenic peptide conjugates are yet to be defined. Furthermore, the majority of drugs are not directly protein reactive and need bioactivation through drug metabolism to be able to produce reactive intermediates capable of binding to protein covalently (Callan et al., 2009a; Castrejon et al., 2010a; Elsheikh et al., 2011). This is named the prohaptens hypothesis. Chemical/drug modified proteins are then processed inside the cell where they are cleaved and linked with major histocompatibility (MHC) molecules, before being presented and recognised via specific T-cell receptors (TCRs) [Figure 1.3]. Also direct linkage to MHC molecules can occur, or to peptides which are attached already to an MHC molecule. The location of protein modifications (i.e. extracellular or intracellular) and antigen source is supposed to determine the processing form of protein; however this still requires experimental endorsement. Whether a CD4+ or CD8+ response occurs, is ultimately determined by the MHC molecule class that presents the drug-derived antigen. Advanced mass spectrometry technical methods have been developed and used to precisely locate the residues of amino acid that are modified by drugs such as flucloxacillin (Jenkins et al., 2009a), sulfamethoxazole (Callan et al., 2009a) and piperacillin (Whitaker et al., 2011a) demonstrating that these may be highly specific.

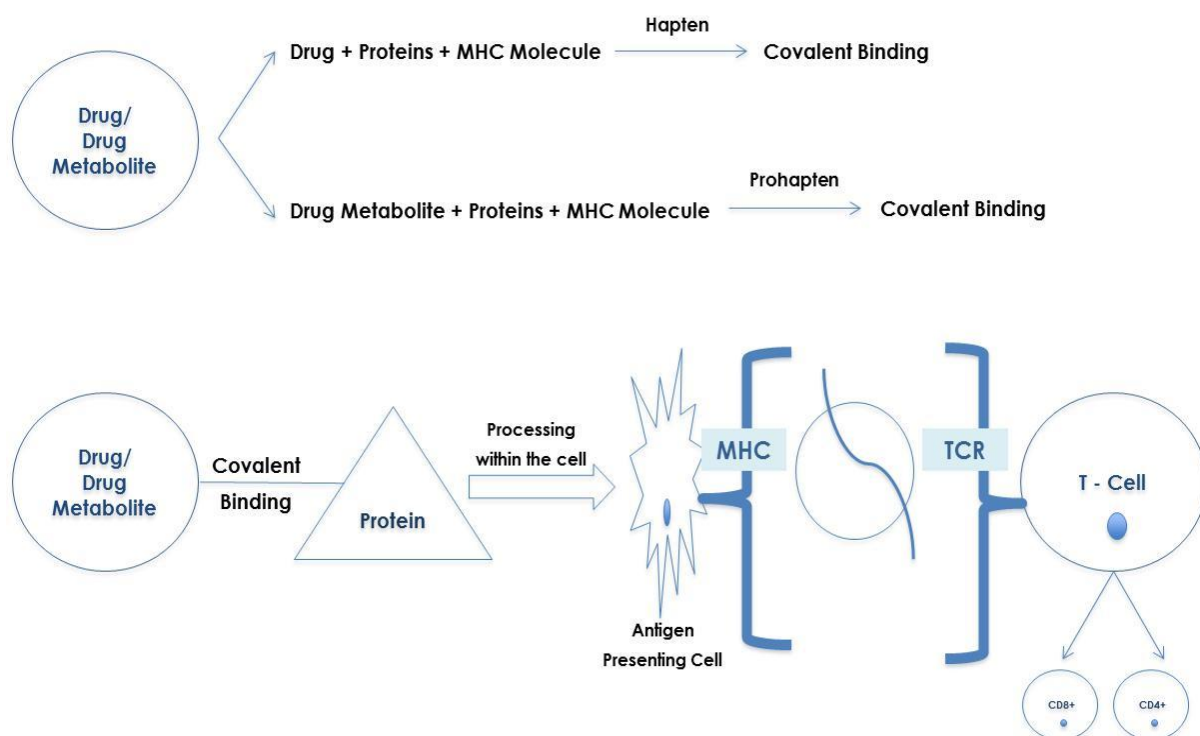


Figure 1.3- Mechanisms of drug-specific T-cell activation (prohaptens/haptens hypothesis). Antigenicity of drug occurs as a result of metabolism and consequent covalent proteins modification of host. Modified proteins of host are then processed inside the cell, cleaved into peptide fragments and linked with MHC molecules then translocated to the cell surface. T-cell receptor will then recognise the MHC/peptide complex. Antigens generated externally (outside of the cell) are processed through an endocytic pathway, attach to MHC class II molecules and stimulate a CD4+ T-cell response. Antigens generated inside the cell are processed through a proteasomal pathway, attach to MHC class I molecules, and stimulate a CD8+ T-cell response [see Figure 1.1].

1.7.6.2 The pharmacological interaction (PI) concept

An alternative mechanism to the hapten hypothesis has been suggested. It has been named the PI concept (the pharmacological interaction of drugs with immune receptors) (Pichler, 2002a). The PI concept describes the capacity of inert drugs to stimulate an immunological response. It is based around results from in vitro assays with human cells from hypersensitive patients that are difficult to explain it with the hapten concept. Pichler and coworkers observed that drugs are presented in MHC molecules expressed on glutaraldehyde fixed antigen presenting cells. These cells which do not process proteins can stimulate T-cell clones the same as non-fixed cells (Zanni et al., 1998). Glutaraldehyde prevents processing of an antigen but conserves their antigen presentation capability. Such findings have been observed by other

laboratories with sulfamethoxazole (Schnyder et al., 1997; Elsheikh et al., 2011), p-phenylenediamine (Jenkinson et al., 2009), lamotrigine (Naisbitt et al., 2003b) and carbamazepine (Wu et al., 2006; Wu et al., 2007). Additionally, rapid Ca^{2+} release from T-cell clones and the internalisation of specific T-cell receptors following drug exposure were discordant with a hapten mechanism (Zanni et al., 1998; Wu et al., 2006). A non-covalent direct association is believed to occur between the drug molecule, and either MHC molecule or TCR [Figure 1.4]. This interaction is readily reversible. Furthermore, as there is *no de novo* primary antigen formation it is suggested that activation through a PI mechanism indicates alloreactivity of the memory T-cell population (Adam et al., 2011).

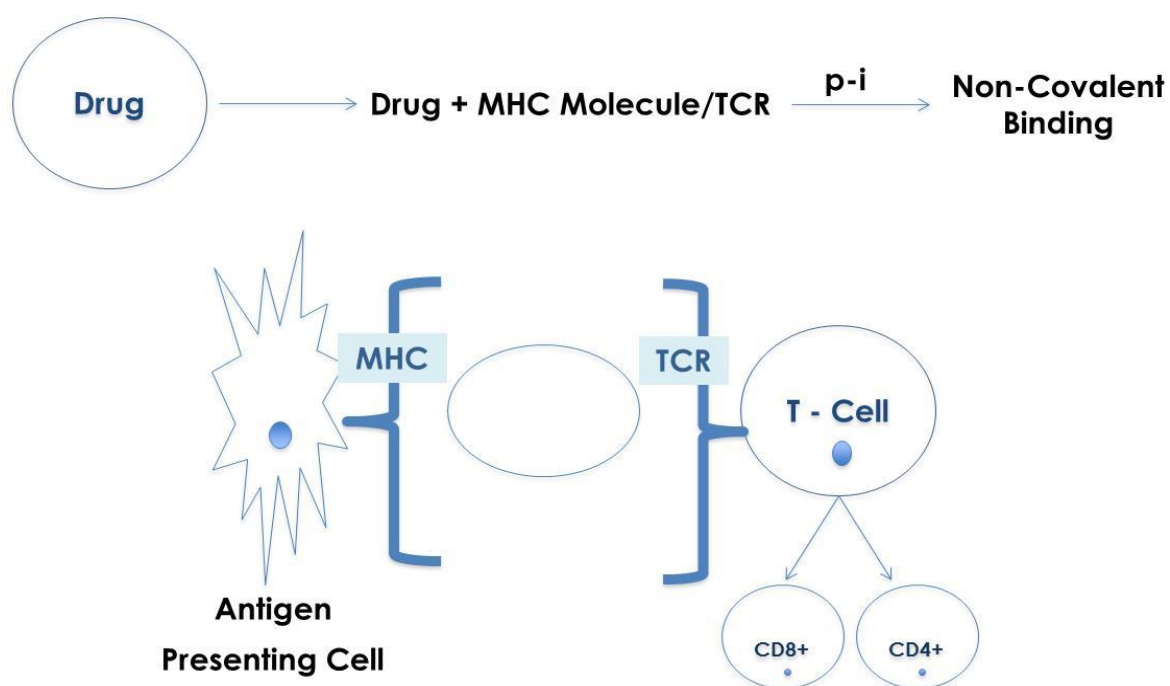


Figure 1.4- Mechanisms of drug-specific T-cell activation (p-i concept). A chemically inert drug can attach non-covalently and directly either to the T-cell receptor or to MHC molecule, stimulating an immune response. This may include hydrophobic interactions, hydrogen bonds or Van der Waals forces.

1.7.6.3 Danger/costimulation hypothesis

Interactions between the innate and adaptive immune system helps to achieve the balance between immune stimulation and regulation. For the host, the first line of defence is the innate immune system where cells recognise non-self-antigens via pattern recognition receptors (PRR) and initiate a danger signal that directs the adaptive immune system through enhanced/altered co-stimulatory signalling. Co-stimulatory signalling is an important element of dendritic cell maturation (Sanderson et al., 2007; Elsheikh et al., 2011). The danger model was described by Matzinger in 1994. It states that immunity is not a simple situation of non-self against self but it is determined by the milieu in which an antigen is presented to T-cells (Matzinger, 1994). Matzinger states that at least two signals are required for immune activation. Signal one is represented by TCR engagement with an MHC-peptide complex. However, alone this is not enough to stimulate an immunological response. The interaction of different co-stimulatory molecules expressed on the T-cell and the antigen-presenting cell (APC) represents signal two. Immune tolerance will occur in the absence of signal two. Signal two can be modulated by several mechanisms. Both, endogenous damage associated molecular patterns (DAMPs) released from dead or damaged cells (e.g. HMGB1, heat shock proteins) and exogenous pathogen associated molecular patterns (PAMPs) (e.g. viral RNA, peptidoglycan, LPS) represent danger signals. Different drugs have been found to initiate danger signalling: (1) amoxicillin promotes a more mature phenotype for dendritic cells (Rodriguez-Pena et al., 2006), (2) abacavir promotes redistribution of heat shock protein in antigen-presenting cells (Martin et al., 2007), and (3) sulfamethoxazole metabolites increases CD40 expression in dendritic cells (Sanderson et al., 2007). However, it should be noted that these studies represent the “tip of the iceberg” in our understand of the role danger signalling play in the development of a hypersensitivity reaction in a susceptible patient. For example, it is not known whether danger signals are provided through infections; hence rendering an otherwise tolerant patient susceptible to a given reaction.

Furthermore, the PI concept states that danger signalling is not needed to promote hypersensitivity reactions. However, experimental evidence to support this is lacking.

1.7.6.4 The terms used to describe small molecules

The following defined terms will be used all through this thesis to illustrate the small molecules interactions with the immune system:

- Immunogen: a molecule that can stimulate an immunological or a cellular response
- Antigen: any molecule that can attach specifically to T-cell receptor or to an antibody
- Prohaptén: a small molecule, which undergo metabolism to be able then to attach to larger macromolecules and stimulate an immune response.
- Haptén: a small molecule that can stimulate an immune response only when attached to a larger macromolecule.
- Co-stimulatory molecule: a molecule that can polarise or induce an immunological response to different antigens.

Several of these characteristics can be exhibited by any drug.

1.7.7 HLA associations and adverse drug reactions

Recent genome-wide association studies reveal that the genetic background of the patient is one of the main risk factors associated with drug hypersensitivity. In 2002, an association between abacavir hypersensitivity syndrome and HLA-B*57:01 was reported (Hetherington et al., 2002; Mallal et al., 2002) and from this time a variety of HLA markers have been identified for both liver (e.g. ximelagatran (Kindmark et al., 2008), flucloxacillin (Daly et al., 2009), lumiracoxib (Singer et al., 2010) and lapatinib (Spraggs et al., 2011)) and skin (e.g. carbamazepine (Chung et al., 2004; McCormack et al., 2011), nevirapine (Martin et al., 2005) and allopurinol (Hung et al., 2005)) reactions [Table 1.4]. Associations are not specifically associated with a

particular compound class or clinical manifestation. However, the associations observed for skin reactions have to date been stronger than those described for liver reactions. Moreover, some associations can be limited to certain ethnic group (Lonjou et al., 2008; McCormack et al., 2011) and this may relate to the various frequencies of risk alleles in various populations.

Genetic association strength can be revealed by calculation of an odds ratio (OR). The value of this ratio compares the frequency of the risk allele in unaffected and affected groups (i.e. patients that have not experienced an adverse reaction and those that have) and can be directly calculated by producing a 2 x 2 table [Figure 1.5]. It provides an indication of effect size.

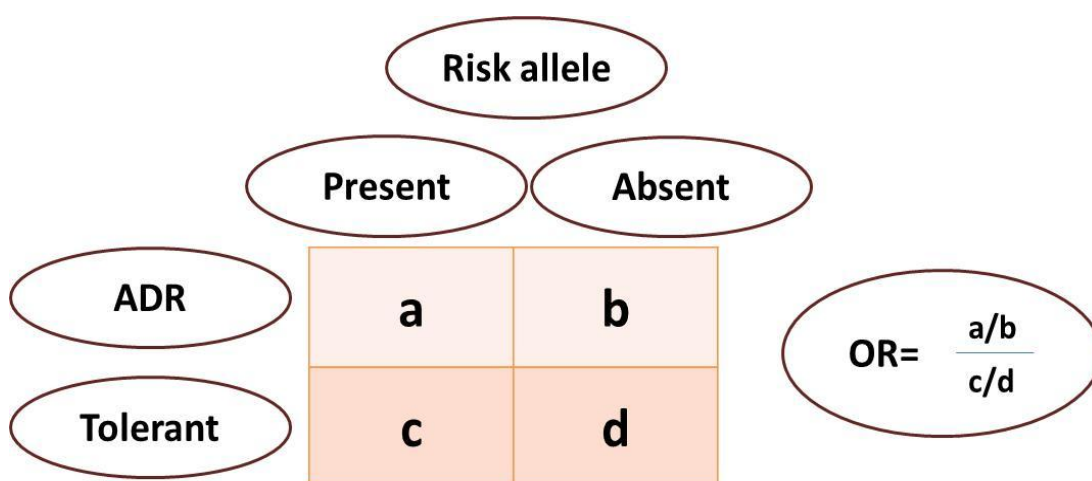


Figure 1.5 – Calculation formula of odds ratios relating the association strength of specific HLA alleles with adverse drug reactions.

Drug	HLA allele	Reaction and ethnicity	Odds ratio (95% CI)	Reference
Drug-induced skin injury (DISI)				
Abacavir	B*57:01	HSS (Europeans)	1945 (110-34352)	(Saag et al., 2008)
Allopurinol	B*58:01	SJS (Han Chinese, Caucasian)	580.3 (34.4-9780.9) 80 (34-187)	(Hung et al., 2005; Lonjou et al., 2008)
Carbamazepine	B*15:02 A*31:01	SJS/TEN (Han Chinese) All phenotypes (Europeans)	1357 (193.4-8838.3) 25.93 (4.93-116.18)	(Chung et al., 2004; Hung et al., 2006; McCormack et al., 2011)
Lamotrigine	B*38	SJS/TEN (Caucasian)	6.8 (2.6-18)	(Lonjou et al., 2008)
Nevirapine	DRB1*01:01	Hypersensitivity (Caucasian)	4.8 (1.3-16.8)	(Martin et al., 2005)
Drug-induced liver injury (DILI)				
Flucloxacillin	B*57:01	DILI (Caucasian)	80.6 (22.8-284.9)	(Daly et al., 2009)
Ximelagatran	DRB1*07:01 DQA1*02:01	DILI (Caucasian)	4.4 (2.2-8.9) 4.4 (2.2-8.1)	(Kindmark et al., 2007)
Lumiracoxib	DRB1*15:01	DILI (Caucasian)	5.3 (3.0-9.3)	(Singer et al., 2010)
Co-amoxiclav	DRB1*15:01 A*02:01	DILI (Caucasian)	2.3 (1.0-5.3) 2.2 (1.6-3.2)	(Hautekeete et al., 1999; O'Donohue et al., 2000; Donaldson et al., 2010; Lucena et al., 2011)
Diclofenac	DRB1*13	DILI (Caucasian)	-	(Daly and Day, 2009)
Clometacin	B*08	DILI	-	(Pariente et al., 1989)
Ticlopidine	A*33:03	DILI (Japanese)	13.0 (4.4-38.6)	(Hirata et al., 2008)
Lapatinib	DQA1*02:01	DILI (Caucasian)	2.2 (1.1-5.7) 9.0 (3.2-27.4)	(Spraggs et al., 2011)
Antituberculosis (isoniazid, rifampicin, pyrazinamide)	DQB1*02:01	DILI (Indian)	1.9 (1.0-3.9)	(Sharma et al., 2002)

Table 1.4- Summary of HLA-associated drug reactions. Adapted from (Yun et al., 2012; Kaniwa and Saito, 2013).

To use these alleles for screening prior to prescription a few considerations must be taken, involving prevalence of the allele, transferability across various populations, and the negative and positive predictive values (Phillips and Mallal, 2010). Nowadays genetic screening for both carbamazepine and abacavir (in Asian ancestry patients) is highly recommended prior to prescription and has been ultimately effective in reducing the incidence of hypersensitivity reactions toward these drugs (Rauch et al., 2006; Phillips and Mallal, 2009). As discussed earlier the physiological function of HLA molecules is to present pathogens-derived-peptides at the surface of the cell to T-cells. Therefore, the discovered genetic associations may indicate a functional consequence of polymorphism contained by the peptide-binding groove. Knowing this, in vitro assays using cells from patients with identified genetic background are needed and have been utilised in this thesis.

In the latest study of induced liver injury by flucloxacillin, more than 80% of patients carried the HLA-B*57:01 allele in comparison to 6% of the control individuals (Daly et al., 2009). But, only 1 in 500 to 1 in 1000 individuals with the risk allele develops DILI when exposed to flucloxacillin despite of the strength of the association. This indicates that if prospective screening is introduced, many patients who may not develop a reaction will be refused treatment (Daly et al., 2009). It has been estimated that more than 13000 patients will require testing to prevent only one case of flucloxacillin-induced liver injury, in comparison to only 13 patients in abacavir hypersensitivity (Phillips and Mallal, 2010). Thus, it seems that the allele is important for the development of hypersensitivity but also other factors are required and yet to be described. This may involve history of concurrent medications or infection and the immune status of the individual. In this case scenario, HLA-typing may function as a diagnostic more than predictive marker test.

Adverse drug reactions including a variety of prescribed drugs and influencing the skin, liver and other organs revealed strong associations with specific HLA alleles. For some reactions, HLA typing before prescription, so that those have the risk allele are not taken the drug associated with the reaction, displays high negative and positive predictive values. The best clinical application associate to the

hypersensitivity reaction is the one induced by abacavir. When this particular reaction is phenotyped in an accurate way, 100% of those who develop it are HLA-B*57:01 positive. Presently, drug regulators worldwide recommend HLA-B*57:01 genotyping prior to abacavir prescription. Severe skin rashes involving toxic epidermal necrosis and Stevens-Johnson syndrome can be induced by carbamazepine and some other anticonvulsant drugs. In particular East Asians, these reactions are highly associated with HLA-B*15:02 and this allele typing is now recommended before carbamazepine is prescribed in these populations. Other HLA allele associations have been defined for skin rash induced by nevirapine, carbamazepine and allopurinol and for liver injury induced by amoxicillin-clavulanate, flucloxacillin, ticlopidine, lapatanib and lumiracoxib. However, the suggestive values for typing HLA alleles linked with these adverse reactions are lesser. Clinical utility thus appears unlikely. Carrying out HLA typing is somewhat complex in comparison to genotyping tests for single nucleotide polymorphisms. With regard to HLA-B*57:01, the methods utilised commonly, involving use of DNA sequencing and sequence-specific oligonucleotide PCR primers are considered, alongside with their effective implementation. Genotyping single nucleotide polymorphisms tagging HLA alleles is an easier substitute to HLA typing but seems inefficiently accurate for clinical usage (Daly, 2014). The drugs flucloxacillin and abacavir will be discussed in greater detail as the identified HLA allele (B*57:01) is associated with both flucloxacillin and abacavir hypersensitivity.

Flucloxacillin is a β -lactam antibiotic used mainly for the treatment of staphylococcal infections. It acts by inhibiting the synthesis of peptidoglycan which is a vital constituent of bacterial cell walls. Even though well tolerated flucloxacillin use is accompanied by the appearance of cholestatic liver injury at an estimated rate of 8.5 in 100 000 new users. Reactions tend to develop in the initial 45 days of treatment (Russmann et al., 2005).

Flucloxacillin metabolism is catalysed by CYP3A4. Flucloxacillin is transformed into a 5-hydroxymethyl metabolite, which has been shown *in vitro* to be toxic to human biliary cells (Lakehal et al., 2001). A substantial increase in lactate

dehydrogenase production was detected, when media from hepatocytes pre-treated with flucloxacillin was added to biliary epithelial cells cultures.

Genome wide associations studies (GWAS) in 2009 found that 84% of patients (43/51) with flucloxacillin-induced liver injury expressed the HLA-B*57:01 allele, in comparison to only 5% of the control population (Daly et al., 2009). The identified HLA allele (B*57:01) is also associated with abacavir hypersensitivity. Therefore, this association may indicate the possibility of T-cells involvement in the reaction pathogenesis. Certainly flucloxacillin positive LTTs have been recorded previously (Maria and Victorino, 1997). However, the causes of liver injury are yet to be defined. It has been found that both flucloxacillin and the 5-hydroxymethyl metabolite bind covalently to albumin at very similar amino acid residues (Jenkins et al., 2009a). This has been illustrated both *in vivo* and also *in vitro*, where modification could be observed in all patients' plasma.

Abacavir is commonly used in a combination therapy for human immunodeficiency virus (HIV) treatment, as it is a nucleoside reverse transcriptase inhibitor (NRTI). Abacavir is an analogue of carbovir which was developed to decrease the poor solubility and toxicity accompanied by carbovir (Daluge et al., 1997).

Abacavir is a guanine analogue and is able to reduce viral reverse transcriptase via chain termination of the developing viral RNA, and competition. Abacavir is a prodrug and needs intracellular phosphorylation, like other NRTIs, to form the active drug phosphorylation occurs in consecutive steps, where abacavir is firstly catalysed by adenosine phosphotransferase (Faletto et al., 1997). Viral reverse transcriptase is ultimately inhibited by carbovir triphosphate (Faletto et al., 1997).

Abacavir hypersensitivity reactions were noticed early clinical developmental trials (Saag et al., 1998). Symptoms included skin rash, fever and nausea that were detectable 6 weeks after starting treatment (Hetherington et al., 2001). Two

independent groups in 2002 reported susceptibility to abacavir hypersensitivity resulted from an association with HLA-B*57:01 (Hetherington et al., 2002; Mallal et al., 2002). The benefit of screening for the risk allele prior to prescription was established by Mallal et al. in 2008. A double-blind long-scale randomised prospective study revealed that 100% of skin test diagnosed hypersensitive patients' express B*57:01 (Mallal et al., 2008). Since these ground breaking studies, screening has been conducted globally leading to an ultimate reduction in the incidence of abacavir hypersensitivity (Phillips and Mallal, 2009).

The immunological basis of abacavir hypersensitivity is well established. Abacavir induces the specific production of pro-inflammatory cytokines, including TNF- α (Martin et al., 2004) and IFN- γ (Almeida et al., 2008) from hypersensitive patients' peripheral blood mononuclear cells, *in vitro*. Skin biopsies obtained from inflamed skin of hypersensitive patients reveal a significant infiltration of CD8+ T-cells, proposing that it is cytotoxic T-cell activity that causes tissue damage (Phillips et al., 2002). Following abacavir exposure, antigen-presenting cells are activated via Hsp70 redistribution; this resulted in the suggestion that the abacavir may stimulate signalling pathways resulting in efficient antigen presentation in individuals with the HLA-B*57:01 allele (Martin et al., 2007).

Recently, innovative studies by Chessman et al. (2008) defined the role of HLA-B*57:01 in the pathogenesis of the disease. Using lymphocytes from healthy volunteers expressing HLA-B*57:01 they described the highly selective binding of abacavir to amino acid residues located deep in the peptide binding cleft (Chessman et al., 2008). Illing et al. and others observation solved the crystal structure of the HLA-B*57:01 abacavir peptide binding interaction and proposed a completely novel mechanism of drug-specific T-cell activation (Illing et al., 2012). They were able to show by mass spectrometry that abacavir binding alters the conformation of the peptide binding cleft and as such the repertoire of peptides that are ultimately displayed on the surface of antigen presenting cells. They propose that these so called "altered-self" peptides and not abacavir provide signals to activate T-cells and ultimately abacavir hypersensitivity. However, it must be noted that the ability of

such peptides to stimulate T-cells in a B*57:01 restricted fashion has not been described.

1.8 Adverse drug reactions without HLA association

1.8.1 Piperacillin

Piperacillin is a piperazine ampicillin derivative (Oh et al., 2009). By the end of 1970s, the drug was launched following thorough research on its antimicrobial efficacy compared with other available antibiotics. Piperacillin is effective against resistant microbes such as *Pseudomonas aeruginosa* and *Klebsiella pneumonia* (Jones et al., 1977; Ueo et al., 1977; Wise, 1977; Dickinson et al., 1978; Verbist, 1978). *In vitro* experiments on bacterial cultures revealed a synergistic effect between aminoglycosides and piperacillin against *Pseudomonas aeruginosa*. Moreover, piperacillin activity against β -lactamase producing resistant bacteria (e.g. *Escherichia coli*, *Haemophilus influenza*, *staphylococci*) can be improved by combining piperacillin with tazobactam (Speich et al., 1998; Sweetman, 2009).

Our investigation focussed on patients with a history of allergic reactions to piperacillin, aztreonam and meropenem. Piperacillin is a member of the penicillin class of antibiotics, which contains β -lactam and thiazolidine ring structures [Figure 1.6]. Meropenem, a carbapenem, contains similar ring structures but the sulfur in the thiazolidine ring has been replaced with a carbon atom, and unsaturation has been introduced. Finally, aztreonam, a monobactam, contains an unfused β -lactam ring.

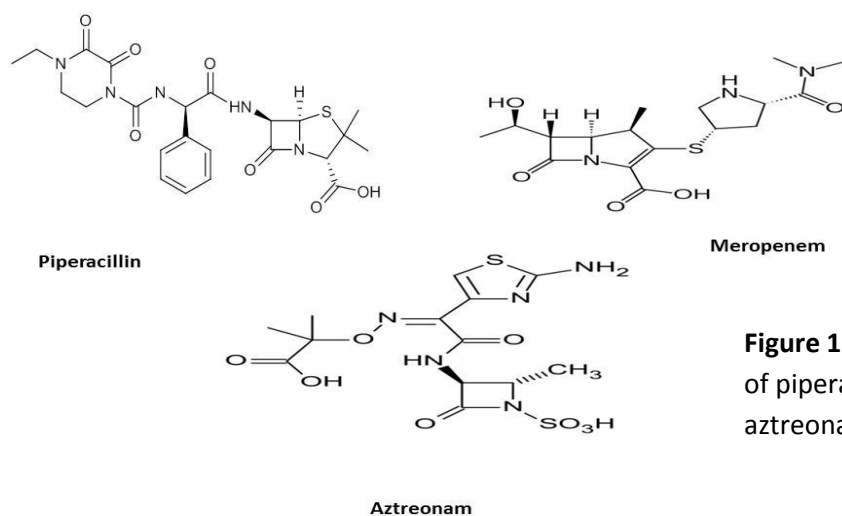
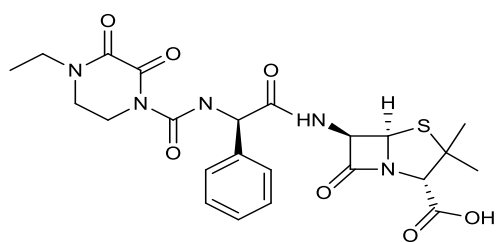


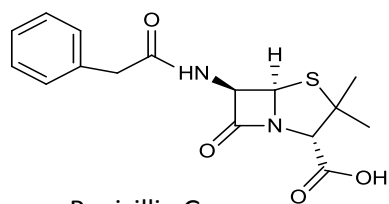
Figure 1.6 - Chemical structures of piperacillin, meropenem, and aztreonam.

Piperacillin is administered as a sodium salt by intravenous or intramuscular injection as it is poorly absorbed from the intestine. The usual piperacillin dosage is 3-4g every 4 or 6 hours, while the maximum daily dose for uncomplicated or mild infections is at 100 to 125 mg/kg. However, adults can be given piperacillin at 200 to 300 mg/kg in divided doses daily intravenously if they are suffering from complicated or serious type of infections (Sweetman, 2009). Approximately 20% of piperacillin in the blood circulation is attached to plasma proteins. Only 20% of piperacillin is excreted unchanged in bile, whereas 60% to 80% of a dose is excreted unchanged in the urine within 24 hours (Tjandramaga et al., 1978; Sweetman, 2009). *In vivo* duodenal samples and *in vitro* studies utilising microsomes of human liver revealed that a small piperacillin fraction is metabolised in the liver in to desethyl-piperacillin and a glucuronide conjugate of the desethyl metabolite. Both desethyl-piperacillin and desethyl-piperacillin glucuronide are detected in human bile and urine (Minami et al., 1991; Komuro et al., 1997; Ghibellini et al., 2006; Ghibellini et al., 2007). Moreover, piperacillin and desethyl-piperacillin are believed to form adducts with proteins, which may act as a functional antigens leading to piperacillin hypersensitivity reactions (Whitaker et al., 2011a).

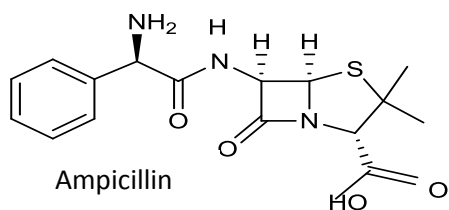
Multiple reports were published in the mid-1980s describing the high incidence of piperacillin reactions, particularly fever in patients with CF (Brock and Roach, 1984; Moss et al., 1984). However, the manufacturers' suggested that mostly patients with CF are liable to hypersensitivity reactions and to support their view they cited reactions with other semisynthetic penicillins including carbenicillin (Sweetman, 2009). Therefore, throughout this thesis piperacillin clones were tested against other structurally related β -lactams [Figure 1.7].



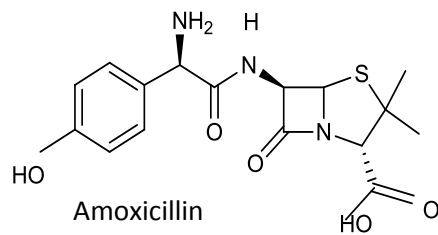
Piperacillin



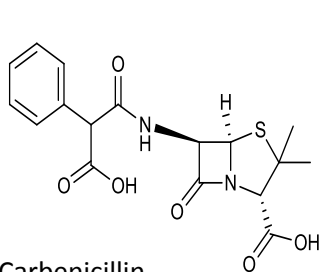
Penicillin G



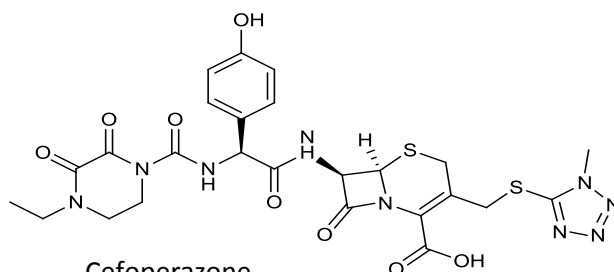
Ampicillin



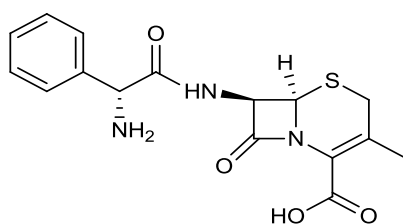
Amoxicillin



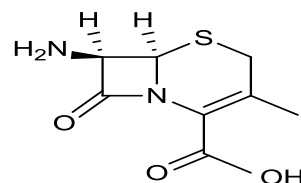
Carbenicillin



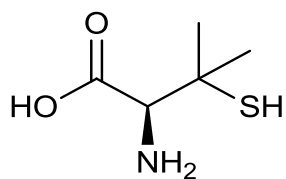
Cefoperazone



Cefalexin



7-Amino-desacetoxycephalosporanic acid



D-penicillamine

Figure 1.7 -Structurally related drugs to piperacillin.

To investigate drugs of our interest, cloning method was used throughout this thesis to generate enough numbers of T cell clones for further studies. T lymphocytes are a pool of cells with exceedingly different characteristics. Firstly, each cell that drives from a specific precursor has reordered its genes for the T cell receptor (TCR) in a different manner from other cells. Therefore, there is a great heterogeneity of T cells in peripheral blood resulted from different TCR expression by the cells that distinguish different epitopes. Additionally, T cells can be further divided in isolated subsets, which are differentiated by different molecules expression, such as CD8, CD4, CD45R and CD25. Such molecules are variably related to the function that a single T cell is intended to accomplish or to the differentiation stage in which at a certain time that cell belongs. Furthermore, the pool of peripheral cells also involves T lymphocytes with variable capacity to release function-associated cytokines. By analysing peripheral blood mononuclear cells (PBMC) many studies on T cell functions can be performed, however since PBMCs cells including a heterogenous population, in many experiments it might be difficult to attain a clear-cut data and to attribute a define function to a defined T cell population. The accessibility of T lymphocytes population that originate from the same progenitor (clone) with a distinctive function and phenotype may be of a great benefit. Oppositely, limitation on the results interpretation should be defined also when a clone is utilised. A clone will comprise cells that share similar phenotypic and functional characteristics, but they may not be demonstrative of the whole T lymphocytes population in *in vivo* settings (Mariotti and Nisini, 2009).

One of the important sources for T cell clones is isolation of PBMC from patients with or shortly recovered from a specific disease, which is related to an antigen-specific T cell expansion (Mariotti and Nisini, 2009). The next two figures illustrate the methods used throughout this thesis to study T-cell responses to antigen using both volunteers and patients' blood samples [Figure 1.8; Figure 1.9].

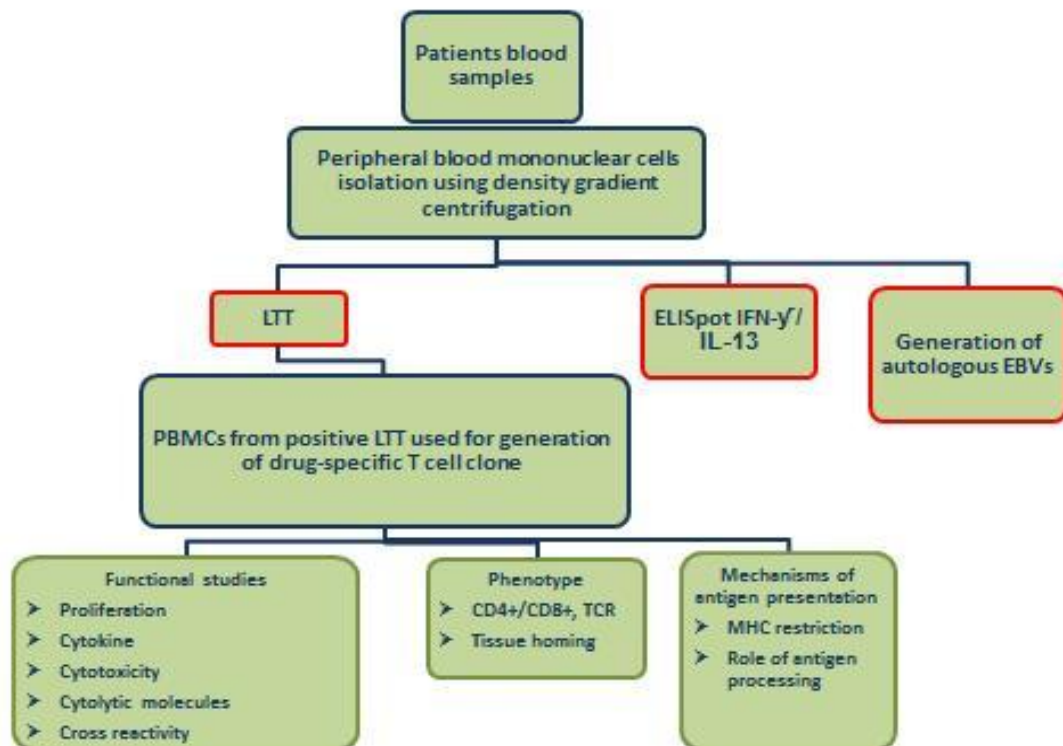


Figure 1.8- A schematic illustration to the methods used to study T-cell responses to antigen using patients' blood samples.

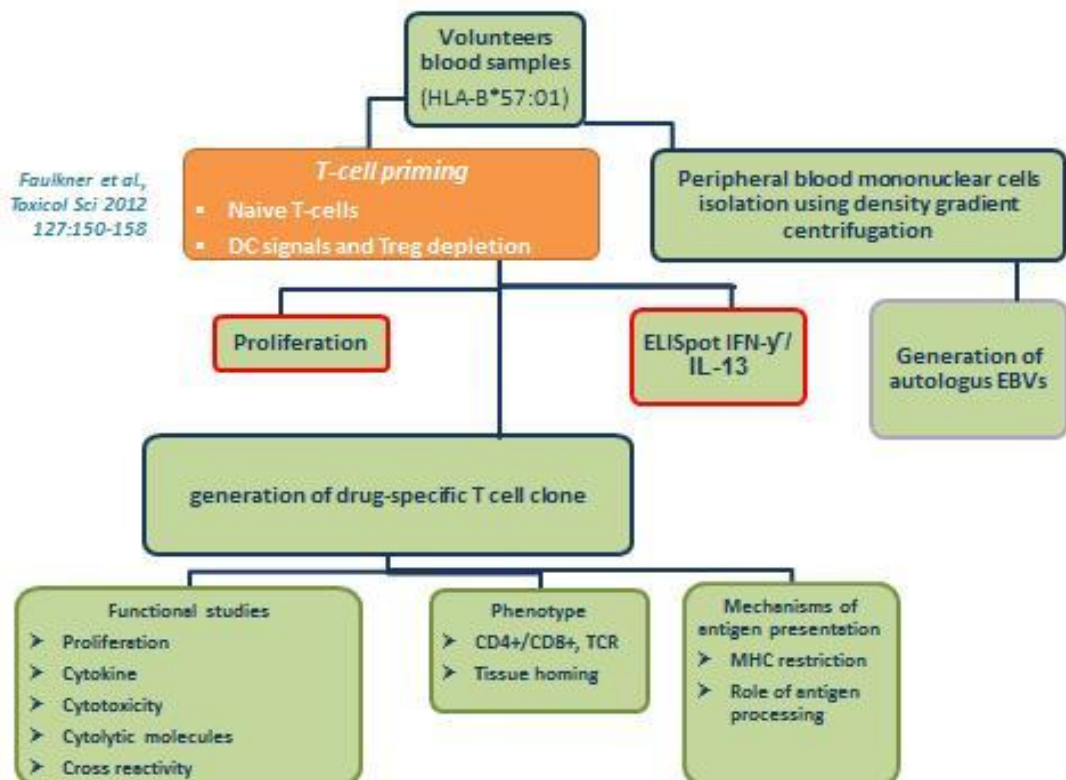


Figure 1.9 - A schematic illustration to the methods used to study T-cell responses to antigen using volunteers' blood samples.

1.9 Aims of the thesis

β -lactam hypersensitivity remains one of the most common causes of immune-mediated drug hypersensitivity. Furthermore, β -lactam antibiotics can induce both skin and liver injury. The incidence of hypersensitivity reactions to β -lactams is three times (28%) greater in CF patients in comparison to patients without CF (8%). The incidence of piperacillin reactions is the highest amongst all β -lactam antibiotics (30-50%) (Parmar and Nasser, 2005; Burrows et al., 2007).

Flucloxacillin is a β -lactam antibiotic used mainly for the treatment of staphylococcal infections. Even though well tolerated flucloxacillin use is accompanied by the appearance of cholestatic liver injury at an estimated rate of 8.5 in 100 000 new users. GWAS in 2009 found that 84% of patients with flucloxacillin-induced liver injury expressed the HLA-B*57:01 allele. To date, there are no studies characterising the drug-specific immune response underlying both piperacillin and flucloxacillin hypersensitivity.

The primary objective of this thesis was to study the role of T lymphocytes in patients with β -lactam mediated skin and liver injury. Secondary objectives were (1) to investigate the mechanisms of drug antigenicity and immunogenicity and (2) to determine whether the HLA associations linked to drug-induced liver injury relate to the HLA-restricted presentation of the drug-derived antigen.

The two drugs piperacillin and flucloxacillin were selected as (1) their use in humans is associated with a high incidence of skin and liver injury, respectively and (2) large number of patients to promote the research.

Chapter Two

The role of drug-specific T-cells in piperacillin hypersensitive patients

2.1 Introduction	51
2.2 Aims	54
2.3 Patients and methods	54
2.3.1 Patients' demographics	54
2.3.2 Chemical and reagents	54
2.3.3 Cell culture medium	58
2.3.4 Isolation and storage of peripheral blood mononuclear cells from venous heparinised blood	58
2.3.5 In vitro enrichment of drug-specific T-cells	59
2.3.5.1 Lymphocyte transformation test	59
2.3.5.2 Enzyme-linked immunospot (ELISpot) assay	60
2.3.5.3 Measurement of cytokine and chemokine secretion using LUMINEX	61
2.3.6 T-cell cloning	64
2.3.6.1 Generation of piperacillin-specific T-cell clones	64
2.3.6.2 Generation of autologous antigen-presenting cells	65
2.3.6.3 Antigen-specificity of T-cell clones	65
2.3.6.4 Proliferation assay	66
2.3.6.5 Phenotypic characterisation of piperacillin-specific T-cell clones	66
2.3.6.6 Chemical cross-reactivity	67
2.3.6.7 Detection of cytotoxic activity of piperacillin-specific T-cell clones	67
2.3.6.8 Secretion of cytokines and cytotoxic molecules	69
2.3.7 Statistical analysis	69
2.4 Results	69
2.4.1 Lymphocytes response from piperacillin-tolerant and piperacillin-hypersensitive patients to piperacillin	69
2.4.2 Specificity and functionality of piperacillin responsive T-cell clones	73
2.4.2.1 Piperacillin specific T-cell clones proliferative response	75
2.4.2.2 Cytotoxicity of piperacillin stimulated T-cell clones	78
2.4.2.3 Secretion of cytokine by piperacillin T-cell clones generated from piperacillin hypersensitive patients	81
2.5 Discussion	83

2.1 Introduction

Cystic fibrosis (CF) is a recessively inherited disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Caucasians have been reported to have the highest incidence of CF (1/2415 of live births in United Kingdom) (Dodge et al., 1997; Kirkby et al., 2009). In 1985 the disease was linked to the long arm of chromosome 7 (Tsui et al., 1985; Wainwright et al., 1985); subsequently the gene sequence which encoded a 1480 amino acid protein called the cystic fibrosis transmembrane conductor regulator (CFTR) was discovered (Kerem et al., 1989). To date over 1,900 mutations have been reported and the number continues to increase. CFTR functions as a cAMP-activated ATP-gated anion channel that transports chloride across epithelial cell membranes. Defective CFTR results in reduced chloride secretion across the apical airway epithelial membrane and increased sodium absorption basolaterally. This results in reduced airway surface liquid and defective ciliary function (Matsui et al., 1998). These thick viscid secretions provide a favourable environment for bacterial infection or colonisation. Ultimately this leads to frequent pulmonary exacerbations, airway destruction and bronchiectasis (Turcios, 2005). Respiratory failure and death is the outcome in 90% of CF patients (Dodge et al., 1997; Amos, 2007; Kirkby et al., 2009). Although it has been well recognised and established that impaired CFTR function influences adversely the secretory epithelium, the CFTR role in non-epithelial cells has received less consideration, or investigators faced difficulties investigating it (Mueller et al., 2011). Surely, if the CFTR alters the immune cells function, it must be expected to lead to an abnormal immune response. Throughout the years the CFTR expression in lymphocytes has been well characterised, however the physiological significance of CFTR expression in lymphocytes is not clear. Some studies demonstrated its importance in volume regulation and cytolysis regulation of CD8 T cells. Other studies suggested that stimulation of Cl^2 currents by CFTR in response to nitric oxide through a cyclic GMP-dependant mechanism is abnormal in T cells in patients with CF. A functional role for CFTR in lymphocytes has been documented by using electrophysiological studies, which recorded cyclic AMP-dependent Cl^2 currents defect in lymphocytes from patients with CF. further studies of CFTR-deficient lymphocytes showed an activation of intracellular Ca^{2+} flux in response to TCR activation (Mueller et al., 2011). Thus the existence and function of CFTR within

lymphocytes has been demonstrated. To date, it has been hard to differentiate abnormal immune response observed in CF patients from the disease phenotype conveyed by epithelial cell dysfunction (Mueller et al., 2011).

Presently, we are seeing a more wide range of clinical disease caused by CF since the advancement of genetic testing. The sweat test (pilocarpine iontophoresis) remains a significant diagnostic test of dysfunctional CFTR (O'Sullivan and Freedman, 2009; Hurt and Bilton, 2012). With regard to chloride concentration normal values are lower than 30mmol/litre. Patients with CF usually have readings more than 60mmol/litre. However, some patients have a borderline range of 30-60mmol/litre. In such patients, nasal potential difference (NPD) testing is utilised to revealed CFTR dysfunction. NPD measures the transepithelial potential variance resulted from the transport of charged ions across nasal membranes (Hurt and Bilton, 2012).

The most resistible microbe colonising in the respiratory passages of patients with CF is *Pseudomonas Aeruginosa* (PA) (Stead et al., 1985; McKone and Aitken, 2004). One of the antibiotics that is highly effective against PA is piperacillin, an antipseudomonal semisynthetic penicillin (Jones et al., 1977; Bodey and Le Blanc, 1978). A β -lactamase inhibitor tazobactam is synergistically used with piperacillin to enhance its antimicrobial activity (Jones et al., 1979). However, due to the development of frequent hypersensitivity reactions, use of the drug combination is limited (Stead et al., 1985). The majority of reactions have a delayed onset of 1-2 weeks (Pleasant et al., 1994). Usually, the reactions involve fever, arthritis and maculopapular or urticarial skin rashes (Pleasant et al., 1994; Wills et al., 1998). The incidence of hypersensitivity reactions to β -lactams is three times (28%) greater in CF patients in comparison to patients without CF (8%). The incidence of piperacillin reactions is the highest amongst all β -lactams (30-50%) (Parmar and Nasser, 2005; Burrows et al., 2007).

The significant increase in survival rate in patients with CF over the past 70 years is mostly related to the better understanding of the role that chronic airway infection plays in the pulmonary disease pathogenesis. This has resulted into the development of new anti-infective treatment strategies to support the control of inflammation,

infection and progressive destruction of the airways (Kirkby et al., 2009). Advancement in genetic technology has many advantages such as assist in tailoring treatment regimens to patients with CF and makes current antibiotic therapies more effective, with less treatment burden and less potential for adverse effects. Furthermore, it is important to mention that recent advancement in pharmaceutical design and molecular genetics have led to the development of agents that can modify dysfunction and correct the underlying pathogenesis of pulmonary disease in patients with CF (Kirkby et al., 2009).

T lymphocytes are believed to play a central role in the pathogenesis of drug-induced skin reactions in patients without CF. Traditionally, CD4⁺ T-cells support the humoral component of the immune response via the release of cytokines, whereas CD8⁺ T-cells mediate tissue injury directly through release of cytotoxic molecules (granzyme B, perforin, granulysin or FasL) (Schnyder et al., 1998; Yawalkar et al., 2000; Pichler, 2002b; Naisbitt et al., 2007; Pichler, 2007; Martin et al., 2010). However recent studies challenge this classification. Drug-responsive CD4⁺ T-cells have been isolated from skin and blood of patients hypersensitive to drugs such as carbamazepine and sulfamethoxazole (Schnyder et al., 1997; Naisbitt et al., 2003a; Wu et al., 2007; Elsheikh et al., 2011) and shown to exert cytolytic activity. Furthermore, by characterising the phenotype and function of T-cells isolated from patients with different forms of cutaneous drug hypersensitivity reactions it has been possible to develop a classification system based on cellular pathophysiology.

In patients with CF, drug hypersensitivity is considered as a major health problem. Hypersensitivity increases patients' morbidity and limits the choice of antibiotics, which is already restricted by increasing microbes' resistance. As the role of T-cells in hypersensitivity in patients with CF has not been defined, 28 patients with piperacillin hypersensitivity and 5 drug exposed tolerant controls were recruited, lymphocytes isolated and antigen-specific T-cell responses analysed. The double-allele deletion of phenylalanine in the CFTR protein position 508 (Δ F508/ Δ F508) was found in 80% of the tolerant and hypersensitive patient groups (O'Sullivan and Freedman, 2009; Hurt and Bilton, 2012). Maculopapular exanthema (MPE) was the form of skin reaction in the hypersensitive patients. Flu-like symptoms, fever and

arthralgia were other clinical sings. The duration since the reaction first occurred ranged between 2-8 months, and the mean time to reaction onset was 6.3 days [Table 2.1].

2.2 Aims

- Characterise the specificity and functionality of T cells by generating piperacillin-specific T cell clones from hypersensitive patients with CF.
- Investigate T cells structural specificity by analysing additional β -lactam antibiotics and structurally related compounds.

2.3 Patients and methods

2.3.1 Patients' demographics

The medical records of around 375 cystic fibrosis patients who are receiving treatment in the Leeds Regional Adult Cystic Fibrosis Unit were included in the study and analysed. Approximately 25% of the patients had experienced a hypersensitivity reaction following exposure to piperacillin. Reactions occurred after intravenous drug administration for at least 48 hours. For this study, blood samples were collected from five piperacillin-tolerant patients who did not show any sign of an adverse reaction following treatment, and eight patients with a history of piperacillin hypersensitivity [Table 2.1]. At the time of the experimental work the mean age in the piperacillin-hypersensitive group was 23.7 years (20-30 years).

2.3.2 Chemicals and reagents

Foetal bovine serum (FBS) was obtained from Invitrogen, Paisley, UK. Human AB serum was obtained from Innovative Research (Michigan, USA). Lymphoprep was bought from Axis Shield (Nycomed, Dundee, UK). Tetanus toxoid (TT) was purchased from the Statens Serum Institute, Copenhagen, Denmark.

Interferon- γ and Interleukin-13 ELISpot kits including substrate and antibodies solution were bought from Mabtech, Stockholm, Sweden. The Fas ligand ELISpot kit was purchased from Abcam (Cambridge, UK). CD8-FITC and CD4-PE antibodies were obtained from BD Bioscience, Oxford, UK. Bio-Plex Pro Human Cytokine Th1/Th2 Panel was bought from Bio-Rad, Hertfordshire, UK. Recombinant human interleukin-2 (rhIL-2) was purchased from Peprotech, London, UK. [^3H]-Thymidine and [^{51}Cr]-Chromium were bought from Moravek (California, USA) and Perkin Elmer Life Sciences (Cambridge, UK), respectively.

Cefalexin was obtained from Fluka Analyticals (Gillingham, Dorset, UK). All other reagents were purchased from Sigma-Aldrich, Gillingham, Dorset, UK.

Patient ID	Age (y)	Sex	Details of the reaction	Sputum classification	Time to reaction	Time since reaction	Courses before reaction	LTT
Piperacillin-tolerant patients					(days)	(years)		-
C1	25	M	Tolerant	Ch. PA				-
C2	22	F	Tolerant	Ch. PA				-
C3	18	F	Tolerant	Non PA				-
C4	27	F	Tolerant	Ch. PA				-
C5	26	F	Tolerant	Inter. PA				-
Piperacillin-hypersensitive patients								
P1* [§]	21	M	MPE, fever	Ch. PA	9	7	3	+++
P2*	24	M	MPE	Ch. PA	11	5	9	+++
P3*	30	F	Fever, eosinophilia	Inter. PA	7	2	14	++
P4* [§]	22	M	MPE, fever	BC	2	1	4	++++
P5*	22	F	Arthralgia	Non PA	5	5	4	++++
P6* [§]	26	M	MPE	Ch. PA	5	2	3	++++
P7*	18	M	Flu-like illness	Ch. PA	2	6	3	++++
P8* [§]	28	F	Fever, arthralgia	Ch. PA	9	6	7	+++
P9	29	F	MPE	Ch. PA	7	12	11	++++
P10	29	M	MPE		3	5	4	++
P11	24	F	Delayed angioedema		6	3	4	-
P12	24	M	MPE		2	5	NA	+++
P13	30	F	Flu-like illness		3	1	9	++
P14	45	F	Fevers		11	5	14	-
P15	22	F	MPE		7	5	6	++
P16	17	M	MPE		4	6	11	-
P17	24	F	Arthralgia MPE		7	2	7	+
P18	22	M	Pruritis		5	4	11	+
P19	31	F	Flu-like illness		9	6	12	+
P20	34	M	MPE		2	10	11	-
P21	32	M	MPE		5	10	8	+
P22	23	F	Fevers unwell		5	3	12	+
P23	29	M	Tight chest		4	6	9	-
P24	18	M	Urticarial rash		2	5	NA	-
P25	40	M	Fevers		10	12	11	-
P26	29	F	MPE		4	5	9	-

P27	34	F	MPE		3	1	17	-
P28	35	F	MPE		7	10	17	-

Table 2.1 – Clinical details of patients with cystic fibrosis. *M*: male, *F*: female, *MPE*: maculopapular exanthema, *Ch. PA*: chronic *Pseudomonas Aeruginosa*, *Non PA*: non-*Pseudomonas Aeruginosa*, *Inter. PA*: intermittent *Pseudomonas Aeruginosa*, *BC*: *Burkholderia Cepacia*, Stimulation index (SI) (+, SI 2-5; ++, SI 5-10; +++, SI 10-20; +++++, SI >20; -, no response), LTT, lymphocyte transformation test; NA, not available. (*)PBMCs of these patients were used in chapter 2, ([§]) PBMCs of these patients were used in chapter 3

2.3.3 Cell culture medium

T-cell medium was consisted of RPMI 1640 supplemented with 10% human AB serum, 1000U/ml penicillin, 0.1mg/ml streptomycin, 2mM L-glutamine, 25mM HEPES and 25µg/ml transferrin.

Antigen-presenting cell medium was consisted of RPMI 1640 supplemented with 10% foetal bovine serum, 1000U/ml penicillin, 0.1mg/ml streptomycin, 2mM L-glutamine and 25mM HEPES.

2.3.4 Isolation and storage of peripheral blood mononuclear cells from venous heparinised blood

Peripheral blood mononuclear cells (PBMCs) were isolated from 70ml of blood, which was collected into heparinised vacutainer tubes. 15ml of blood was layered on top of 10ml of lymphoprep and the density gradient centrifugation (2000 rpm, 25 min, 0 brake, at room temperature) was used for erythrocytes sedimentation. After that the PBMCs band was formed as a buffy coat layer between the lymphoprep layers and the plasma. The buffy coat layer containing the PBMCs was then gently aspirated from the interface with a sterile pasture pipette. PBMCs were washed twice in Hanks balanced salt solution (HBSS) to remove any residual lymphoprep using density centrifugation at 1800 rpm and 1500 rpm, respectively. Cell yield was then resuspended in 10ml HBSS. An aliquot of 10µl was added to 40µl HBSS and then 10µl of this diluted suspension of cell was added to trypan blue (10µl, 0.2% w/v). A 10µl aliquot of the cell suspension/ trypan blue mixture was then placed on a Neubauer haemocytometer (Sigma-Aldrich, Dorset, UK) and counted under a Leica DME microscope (Leica Microsystems, Milton Keynes). Cell viability was assessed by trypan blue [unstained (viable) and stained (non-viable)] and calculated by the following equation: percentage of viable cells = $100 \times (\text{number of viable cells} / \text{total number of cells})$. Cell viability was typically more than 95%. Viable cells were resuspended in culture medium at the required concentration.

Freshly isolated PBMCs were resuspended in a freezing mixture of 90% foetal bovine serum (Invitrogen, Paisley, UK) containing 10% DMSO (sigma-Aldrich, Dorset, UK) at a concentration of 10^7 cells/ml. Cells were dispensed into cryovials (1ml/ vial) on ice. Cryovials were placed in a freezing container called Mr Frosty-style which is filled with 70% isopropanol, and saved in -80°C freezer. After 24 hours the cryovials were all transferred to liquid nitrogen. Cells should be thawed and processed quickly for better viability and recovery. To thaw the cells, warm RPMI was added into the cryovials containing cell suspension followed by transferring to a sterile 50ml tube containing warm RPMI. Cells were centrifuged using density gradient centrifugation for 5 minutes at 1500 rpm then resuspended in the required volume of warm culture medium. Cell viability and number were assessed as described above.

2.3.5 *In vitro* enrichment of drug-specific T-cells

2.3.5.1 Lymphocyte transformation test (LTT)

The lymphocyte transformation test is particularly designed to detect the existence and proliferation of drug-specific memory T-cells in the hypersensitive patients' peripheral blood (Nyfeler and Pichler, 1997). Freshly isolated PBMCs (1.5×10^5 , 100 μl) were cultured with piperacillin (0.06-8mM), or tetanus toxoid (5 $\mu\text{g}/\text{ml}$) as a positive control, in triplicate wells for six days, in 96-well U-bottomed tissue culture plates (37°C , 5% CO_2). Cell cultures containing lymphocytes in the absence of piperacillin were considered as a negative control. [^3H]-Thymidine (0.5 $\mu\text{Ci}/\text{well}$) was added for the final 16 hours of incubation to measure proliferation. Plates were harvested and counted as count per minute (cpm) on a liquid scintillation counter (Wallac microbeta trilux, PerkinElmer, Cambridge, UK). Proliferative responses were calculated by comparing proliferation in drug treated cultures vs. control cultures. Stimulation index ($\text{SI} = \text{cpm in drug treated cultures}/\text{cpm in control cultures}$). An $\text{SI} \geq 2$ was considered as a positive result (Pichler and Tilch, 2004).

2.3.5.2 Enzyme-linked immunospot (ELISpot) assay

Filter plates from Multiscreen_{HTS} (Millipore, Watford, UK) were coated at 4°C overnight with interferon- γ (IFN- γ) and interleukin-13 (IL-13) capture antibodies at (15 μ g/ml) and (10 μ g/ml), respectively. The following day, all wells were blocked with T-cell medium (30 minutes, at room temperature, 200 μ l) after a thorough wash with Hank's balanced salt solution (HBSS) for five times. At the end of incubation, T-cell medium was removed, and responders cells were added to wells ([lymphocytes 5×10^5 , 100 μ l], [T-cell clones 0.5×10^5 , 50 μ l + autologous irradiated antigen-presenting cell 0.1×10^5 , 50 μ l]) and the preferred concentration of the antigen (100 μ l). Medium and phytohemagglutinin (PHA 5 μ g/ml) were then added to corresponding wells. Plates were incubated at 37°C, 5% CO₂, and developed after 48 hours, according to the instructions of the manufacturers. At the end of the incubation cells were removed and wells were washed five times with 250 μ l phosphate buffer solution (PBS). Biotin-labelled detection antibodies specific to IFN- γ and IL-13 were diluted to 1 μ g/ml in PBS containing 0.5% FBS and added to each well (100 μ l). Plates then were incubated for 2 hours at room temperature before wells were washed for 5 times with PBS (250 μ l/well). Streptavidin-ALP was diluted (1:1000) in PBS containing 0.5% FBS and added to wells (100 μ l/well, at room temperature for 1 hour). At the end of the incubation, wells were washed again five times with PBS (250 μ l) and spots were visualised by the addition of the substrate BCIP/NBT at 100 μ l/well and left for 15 minutes until spots appear. All plates then were rinsed gently under tap water and left to dry to be ready for spots counting on an AID ELISpot reader (Cadama Medical, Stourbridge, UK). A schematic illustration of the ELISpot assay is shown in Figure 2.1.

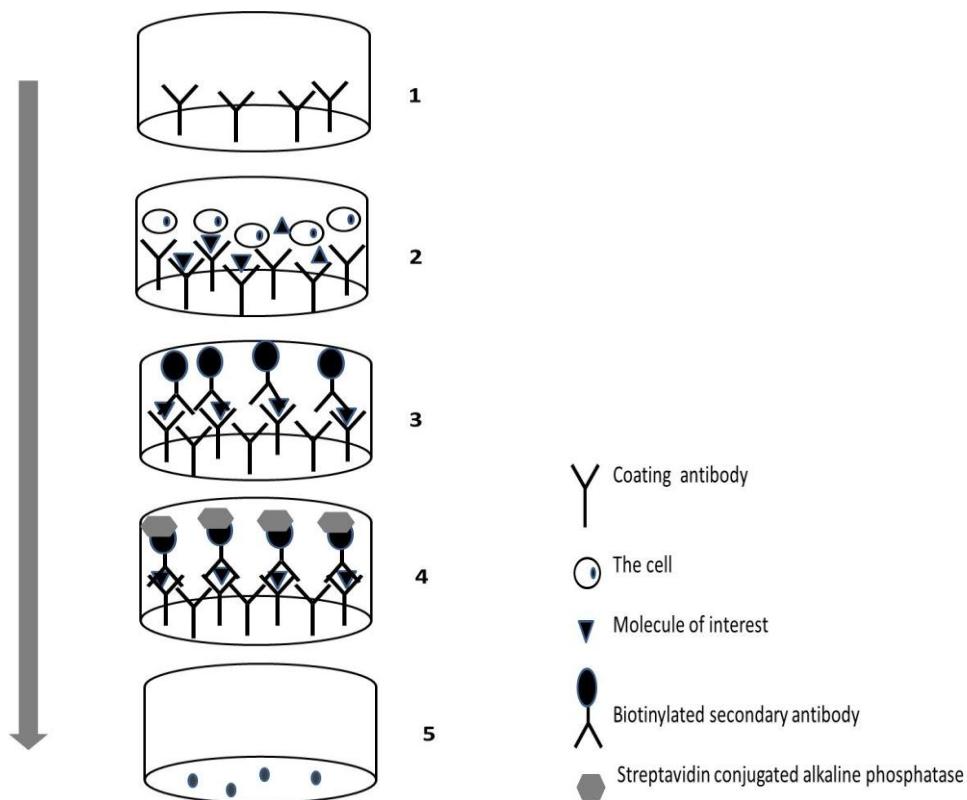


Figure 2.1- Schematic illustration of the enzyme-linked immunospot (ELISpot) assay. (1) 96-well ELISpot plate is coated over night at 4°C with specific antibody for the molecule of interest. (2) The following day cells are added and incubated in the plate for 48 hours and the interested molecule is captured. (3) Biotinylated specific secondary antibody for the molecule of interest is added, after the cells being removed. (4) Streptavidin conjugated alkaline phosphatase is added and it binds to the biotinylated secondary antibody. (5) Finally, alkaline phosphatase will catalyse a colour change reaction, after addition of the substrate solution. This will result in the development of dark distinct spots.

2.3.5.3 Measurement of cytokine and chemokine secretion using LUMINEX

Levels of secreted cytokines (IL-4, IL-5, IL-6, IL-10, IL-13, IL-1 β , IFN- γ , TNF- α , MIP-1 α and MIP-1 β) were analysed and measured using Luminex (xMAP-based platform, Biorad laboratories, Hercules, USA) multiplex biorad bead assay. The Luminex assay allows multiple cytokines to be simultaneously quantified within the same sample (Figure 2.2). Antibodies coupled to colour-coded beads directed at every target molecules, are added and incubated with the sample. Different bead with a distinctive unique colour is attached to each antibody. Reporter labelled with fluorescent, also specific for the target is added later to quantify the amount of

analyte attached to every single bead. The bead colour and the bounded analyte amount are both detected by the Luminex reader.

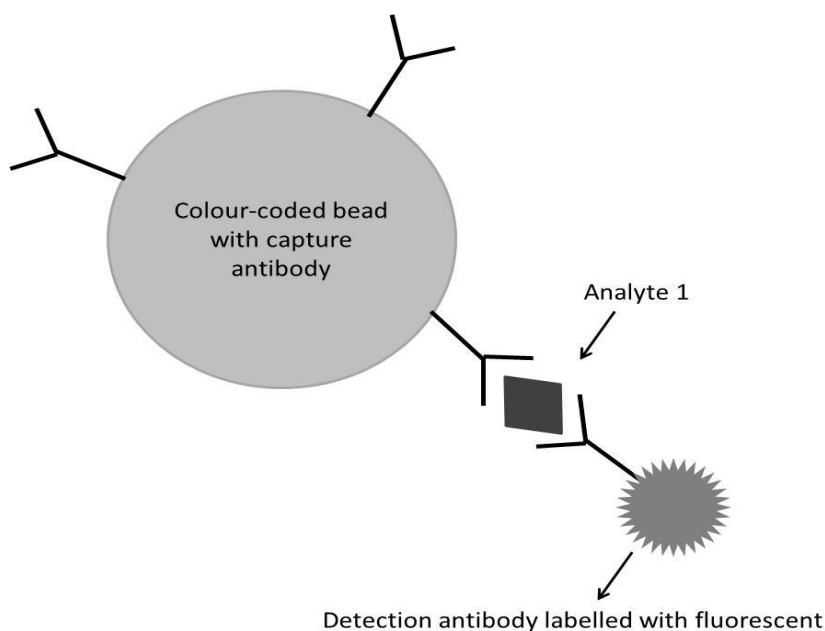


Figure 2.2- Luminex assay principle. Antibodies coupled to colour-coded beads directed at every target molecules, are added and incubated with the sample. Different bead with a distinctive unique colour is attached to each antibody allowing multiple analytes to be investigated within the same sample.

Samples used comprised supernatants of lymphocytes or drug-specific clones' cultures +/- culprit drug. Lymphocytes (1.5×10^5) from five piperacillin hypersensitive patients and seven piperacillin-specific T-cell clones (0.5×10^5 + autologous irradiated EBV-transformed B-cells 0.1×10^5) were incubated in the presence and absence of 2mM piperacillin. After 48 hours supernatants (100µl) were collected from the wells and stored at -80°C until the time of analysis by Luminex. On the day of the Luminex assay, samples were firstly thawed on ice then diluted with sample buffer. One single vial of standards was reconstituted in 250µl water, mixed, vortexed and diluted as shown in Table 2.2.

Standard (pg/ml)	Water to add (μl)	Standard to add (μl)
10,000	250	0
Standard (pg/ml)	Assay buffer to add (μl)	Standard to add (μl)
2000	200	50μl of 10,000pg/ml
400	200	50μl of 2000pg/ml
80	200	50μl of 400pg/ml
16	200	50μl of 80pg/ml
3.2	200	50μl of 16pg/ml

Table 2.2- Serial dilution of standards for Luminex assay.

Analysis of supernatants was performed following the manufacturers' protocol (Linco Research, Inc). The filter plate was prewet with assay buffer (200μl, 10 minutes incubation on plate shaker, at room temperature) followed by assay buffer removal by vacuum. Standards, controls and samples were then added to the appropriate wells in duplicate (25μl/well). Magnetic beads (50μl/well) were also added and incubated in the dark for 30 minutes at room temperature on a plate shaker. After that wells were washed twice before incubation with detection antibody (25μl/well, 30 minutes, at room temperature, on plate shaker). Samples were then incubated again for extra 10 minutes with streptavidin phycoerythrin (25μl/well) under the same conditions. Wells were washed two times with wash buffer before sheath fluid was added (150μl/well, 5 minutes, on plate shaker). The plate was then analysed using Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, CA).

2.3.6 T-cell cloning

Theoretically, T-cell cloning is considered as a simple technique where PBMCs are freshly isolated and seeded at one cell per well then induced with PHA to proliferate. Antigenic specificity of proliferated cells is then experimentally tested. Practically, T-cell cloning method is difficult as many different problems can be encountered. T-cell cloning entails handling of long-term cultures in an aseptic technique which requires a high level of expertise. Moreover, several thousand separate cultures must be generated to establish working numbers of specific T-cell clones, as antigen specific T-cells frequency is incredibly low. Limiting dilution was the procedure used to generate T-cell clones following previously described method (Schnyder et al., 1997; Wu et al., 2006). The aim of T-cell cloning is to generate monoclonal populations of antigen-specific T-cells that survive in the *in vitro* cultures for long enough time to be tested and completely studied.

2.3.6.1 Generation of piperacillin-specific T-cell clones

Freshly isolated PBMCs (1×10^6 ; 500 μ l) from piperacillin hypersensitive patients were incubated in 48-well plates with piperacillin (1, 2mM, 500 μ l) in T-cell medium. Cultures were supplemented on days 6 and 9 with IL-2 (60U/ml) (Peprotech, UK). On day 14, cultures treated with drug were pooled, washed with medium and cells were counted by trypan blue dye exclusion. CD4⁺ and CD8⁺ cells were separated by CD8⁺ positive selection using CD8 Multisort kit (Miltenyi Biotech, Bisley, UK). Cells were harvested and resuspended in HBSS containing 0.5% bovine serum albumin (BSA) and 2mM ethylenediaminetetraacetic acid (EDTA). CD8 magnetic microbeads (20 μ l) were added to the cells, mix well and incubated at 4°C for 15 minutes. The cells were then centrifuged, washed two times and added to an MS column (no. of cells $\leq 10^7$) held in a magnet. Unlabelled, negative cells pass via the column were considered as the CD4⁺ fraction. The MS column was then detached from the magnet and the CD8⁺ cells were promptly flushed out. The cell populations' purity was determined using fluorescence activated cell sorting (FACS) analysis. Phycoerythrin (PE)-labelled CD4 (3 μ l) and fluorescein isothiocyanate (FITC)-labelled CD8 (3 μ l) antibodies were added to the separated cells aliquot and incubated for 20 minutes in the dark at 4°C. A BD FACS

Canto II flow cytometer (BD Biosciences, Oxford) was used for events collection. In general purity was > 95%. Separated cells were then serially diluted following an established method (Wu et al., 2006). Cells were seeded in 96-well U-bottom plates into three different densities 0.3, 1 and cells/well and restimulated with a stimulation cocktail containing allogenic irradiated PBMCs (5×10^4 cells/well), IL-2 (60U/ml) and PHA (5µg/ml). Well-growing clones were marked and transferred to new 96-well U-bottom plates and split as required to 4wells/clone. Every 14 days, clones were restimulated as described above in order to maintain T-cell expansion.

2.3.6.2 Generation of autologous antigen-presenting cells

Epstein-Barr virus (EBV) transformed B-cell lines were generated by transformation of freshly isolated PBMCs using supernatant from the virus-producing cell line B9.58. 5ml of the supernatant was filtered by a 0.45µm syringe filter on to a PBMC pellet containing 5×10^6 cells. 5µl of cyclosporin A (CSA) was then added to prevent T-cell mediated suppression of B-cell infection and to inhibit EBV from inducing T-cell proliferation. After overnight incubation (37°C, 5% CO₂), cells were washed, resuspended at 1×10^6 /ml in antigen-presenting cell medium containing CSA (1µg/ml) and transferred to a 24-well cell culture plate (1ml/well). Cells were fed twice per week with fresh antigen-presenting cell medium and CSA was omitted from the culture medium after 14 days. When confluent observed, cells were transferred to a tissue culture flask.

2.3.6.3 Antigen-specificity of T-cell clones

At least four weeks after serial dilution, piperacillin-specificity was determined by the addition of piperacillin (2mM; 100µl) and autologous irradiated EBV-transformed B-cells (1×10^4 /well; 50µl) to T-cell clones (5×10^4 /well; 50µl) and incubated for 48 hours. [³H]-Thymidine (0.5µCi) was added in the final 16 hours of incubation to measure the proliferation by scintillation counting. Clones that have stimulation index of $2 \geq$ were taken and expanded in IL-2 containing medium by repetitive mitogen stimulation for further analysis.

2.3.6.4 Proliferation assay

The most common technique used to measure proliferation *in vitro* is depending mainly on the [³H]-Thymidine uptake. After two weeks of T-cell clones' restimulation, 5×10^4 T-cells were incubated for 48 hours in duplicate with autologous irradiated EBV-transformed B-cells (1×10^4) in the presence and absence of piperacillin (0.1, 0.5, 1, 2 and 3mM) in a total volume of 200µl in U-bottom 96-well plate. It is useful to do the proliferation assay using a wide range of drug concentrations to find out the best concentration that gives the optimal proliferative response. In the final 16 hour of incubation, [³H]-Thymidine (0.5µCi/well) was added and then cells were harvested by a multiwell harvester to aspirate and transfer them onto a filter paper. Only [³H]-Thymidine labelled DNA were transferred onto the filter paper, while any non-incorporated [³H]-Thymidine in the medium were discarded. The incorporated radioactivity was determined by a liquid scintillation counter (Wallac microbeta trilux, PerkinElmer, Cambridge, UK) as count per minute (cpm). T-cell clones with a stimulation index equal or greater than two were taken to be piperacillin-specific T-cell clones and maintained in IL-2 containing medium in 48-well culture plate. Antigen-induced T-cell response was quantified by both proliferation and measuring cytokine secretion using multiplex technique (see 2.3.5.3) and IFN-γ ELISpot assay (see 2.3.5.2). If multiplex technique was planned, the culture supernatants from each condition were pooled and collected prior the addition of [³H]-Thymidine and stored at -80°C until the day of the assay.

2.3.6.5 Phenotypic characterisation of piperacillin-specific T-cell clones

T-cell clones (1×10^5) were washed and incubated with CD4-PE and CD8-FITC antihuman monoclonal antibodies (3µl) in a 96-well V bottomed plate (at 4°C, 20 minutes) in the dark in HBSS. At the end of incubation, cells were washed three times by centrifuging the plate (at 4°C, 5 minutes, 1500 r.p.m). Finally the pellets of the cells were resuspended in 300µl FACS buffer (HBSS containing 0.5% BSA and 2mM EDTA) and transferred to FACS tubes for flow cytometric analysis on the BD FACS Canto II flow cytometer (BD Biosciences, Oxford).

2.3.6.6 Chemical cross-reactivity

T-cell clones (5×10^4 /well) were incubated with irradiated autologous EBV-transformed B-cells (1×10^4 /well) in the presence and absence of each of the compounds (piperacillin, penicillin G, ampicillin, amoxicillin, carbenicillin, cefoperazone, cefalexin, 7-amino-desacetoxycephalosporanic acid and D-penicillamine; [Figure 1.7] (1, 2 and 4mM) for 48 hours. All cells were seeded in duplicate in 96-well U-bottomed plates and kept in 37°C, 5% CO₂. In the final 16 hour of incubation, [³H]-Thymidine (0.5μCi/well) was added and proliferation determined by scintillation counting. Stimulation index calculated accepting values ≥ 2 as a positive response.

2.3.6.7 Detection of cytotoxic activity of piperacillin-specific T-cell clones

2.3.6.7.1 Standard ⁵¹Cr-release assay

Autologous EBV-transformed B-cells (1×10^6) were centrifuged (1500 r.p.m, 5 minutes) and cell pellet were loaded with ⁵¹Cr (0.5μCi, 50μl) and incubated for 1 hour at room temperature. Cells were then washed thoroughly for three times with 50ml RPMI medium to unincorporated radiation and resuspended in 20ml of culture medium to adjust cell concentration (5×10^4 cells/ml).

T-cell clones were seeded, and incubated with chromium loaded autologous EBV-transformed B-cell, in 96-well U-bottom plate in duplicate in different concentrations to bring effector: target ratio to 25:1 and 50:1 in the presence and absence of piperacillin (2 and 4mM). After 4 hour incubation at 37°C in 5% CO₂, 100μl of the supernatant was collected from each well culture and transferred to polypropylene scintillation vials (Perkin Elmer life sciences, Cambridge, UK) then topped up with 4ml scintillation fluid (Perkin Elmer life sciences, Cambridge, UK). Additional spontaneous and maximum ⁵¹Cr release controls were determined by parallel incubations of ⁵¹Cr labelled autologous EBV-transformed B-cells with medium and methanol in the presence and absence of piperacillin. A total of eight wells replicates were established for each single condition of spontaneous and

maximum release. Piperacillin direct toxicity was excluded by incubating piperacillin with ^{51}Cr loaded autologous EBV-transformed B-cells for four hours in the absence of T-cells. Radioactivity in supernatants was measured using Gamma counter. Cytolytic activity was then calculated using the following formula: percentage of cytolytic activity = [(average of medium or drug treated culture) – (average of spontaneous release)] / [(average of maximum release) – (average of spontaneous release)] x 100.

In case of revised ^{51}Cr -release assay, autologous EBV-transformed B-cells were pulsed for 16 hour with piperacillin or medium. At the end of the incubation, cells were washed extensively for three times to remove unbound drug then loaded with ^{51}Cr as described above.

2.3.6.7.2 Detection of increased cell surface CD107a expression using flow cytometry

T-cell clones (5×10^4 cells/well; total volume 200 μl) were incubated in 96-wll V-bottom culture plate in duplicate, with irradiated autologous EBV-transformed B-cells (1×10^4 cells/well) in the presence and absence of piperacillin for 16 hour. At the end of the incubation, cells were incubated for 20 minutes with FITC-labelled anti-human CD107a monoclonal antibody (BD pharmingen, Oxford, UK) at 4°C. The cells were then washed and resuspended in 300 μl FACS buffer (HBSS containing 0.5% BSA and 2mM EDTA) and transferred to FACS tubes for flow cytometric analysis on the BD FACS Canto II flow cytometer (BD Biosciences, Oxford). The protocol was adjusted to acquire a minimum of 30,000 cells per sample.

2.3.6.8 Secretion of cytokines from piperacillin-specific T-cell clones

Levels of secreted cytokines (IL-4, IL-5, IL-10, IL-13, IL-1 β , IFN- γ , TNF- α and MIP-1 β) from piperacillin-specific T-cell clones were analysed and measured using Luminex assay as described above (see 2.3.5.3).

2.3.7 Statistical analysis

The Mann-Whitney test was used for comparison of proliferation in control vs. drug treated wells.

2.4 Results

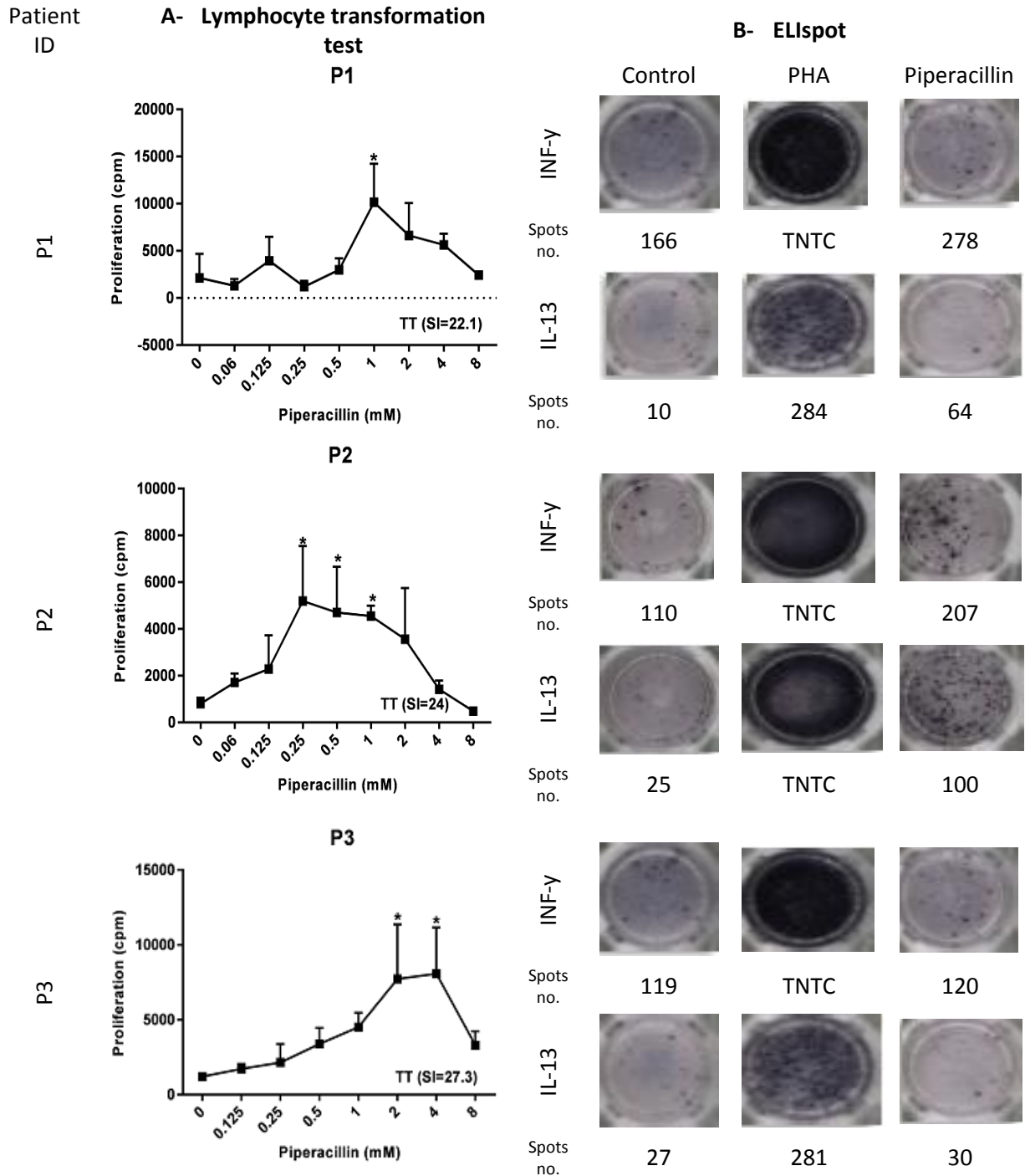
2.4.1 Piperacillin-specific Lymphocytes proliferative responses and cytokine release from tolerant and hypersensitive patients.

Lymphocytes from the 8 piperacillin-hypersensitive patients [Table 2.1] were stimulated to proliferate with piperacillin in a concentration-dependent fashion (0.06-8 mM). Maximum responses were detected at a concentration of 1-2 mM (control: 1526 \pm 2171 cpm, piperacillin 2 mM: 19108 \pm 2957 cpm). Therefore these two concentrations of piperacillin were used in the functional studies described below [Figure 2.3, 2.4].

Lymphocytes from the piperacillin-tolerant patients [Table 2.1] did not proliferate in response to piperacillin. However responses were detected to the control protein antigen tetanus toxoid (TT = 5 μ g/ml) (control: 437 \pm 310 cpm, TT: 5104 \pm 2409 cpm, piperacillin 2 mM: 449 \pm 170 cpm) [Figure 2.4].

ELISpot was used to detect active secretion of the Th1 and Th2 cytokines IFN- γ and IL-13. Lymphocytes from hypersensitive patients, but not tolerant controls, were stimulated to secrete IFN- γ and IL-13 in the presence of piperacillin (2mM) [Figure 2.3]. Furthermore, lymphocytes from both piperacillin tolerant and hypersensitive

patients secreted both cytokines when stimulated with the positive control phytohaemagglutinin (PHA: 5 μ g/ml).



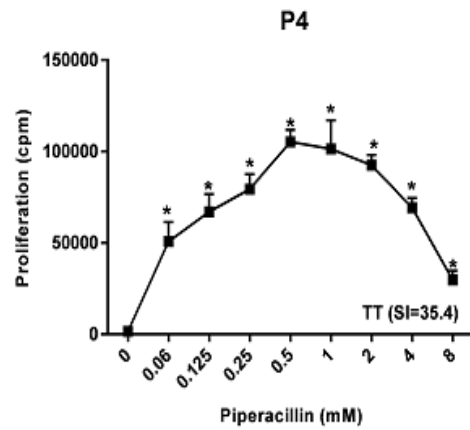
Continue next page

Patient ID

A- Lymphocyte transformation test

B- ELISpot

P4



INF- γ



Spots no.

Control: 122, PHA: TNTC, Piperacillin: 223

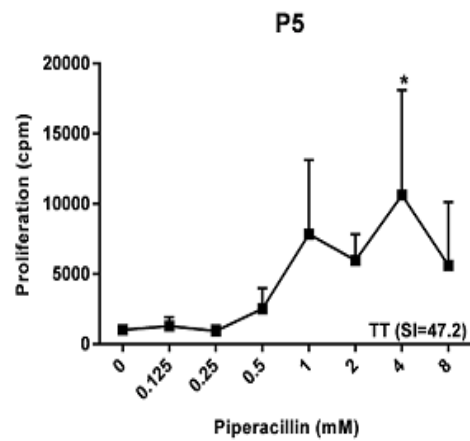
IL-13



Spots no.

Control: 10, PHA: 232, Piperacillin: 268

P5



INF- γ



Spots no.

Control: 38, PHA: TNTC, Piperacillin: 92

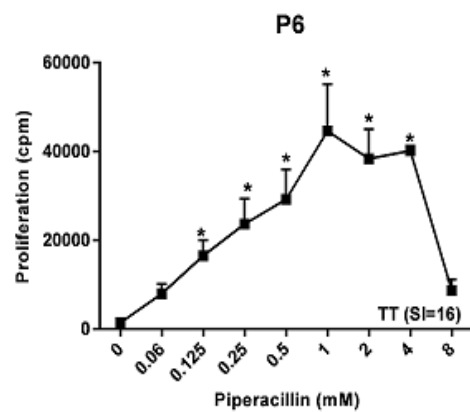
IL-13



Spots no.

Control: 10, PHA: 306, Piperacillin: 9

P6



INF- γ



Spots no.

Control: 59, PHA: TNTC, Piperacillin: 96

IL-13



Spots no.

Control: 4, PHA: 266, Piperacillin: 21

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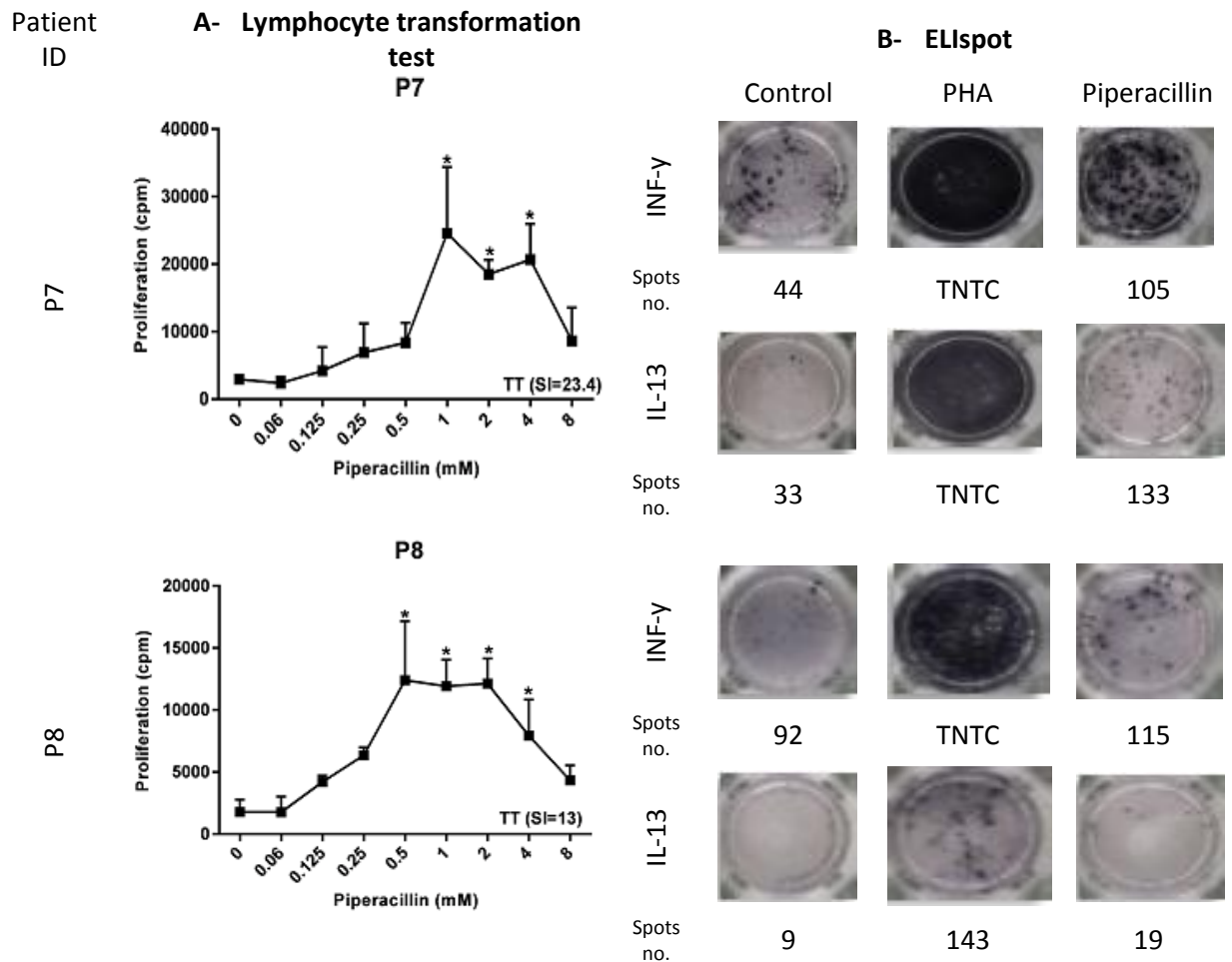


Figure 2.3 – Isolated PBMCs from cystic fibrosis hypersensitive patients' respond to piperacillin as detected by (A) lymphocytes transformation test and (B) ELISpot. **(A)** PBMCs were specifically stimulated with piperacillin (0.06-8 mM) for 6 days. In the last 16h of the incubation, cultures were pulsed with [3 H]-thymidine. Data presented as the average count per minute of triplicate cultures (cpm \pm SD). Tetanus Toxoid was used as a positive control (TT= 5 μ g/ml). (* $p < 0.05$). ; **(B)** (0.5×10^6) lymphocytes were added to wells in the presence and absence of piperacillin (2mM). Plates were incubated at 37 $^{\circ}$ C and 5% CO $_2$ and developed after 48 hours following the manufacturer's instructions. IL-13 and IFN- γ spots were visualised and counted using AID Software.

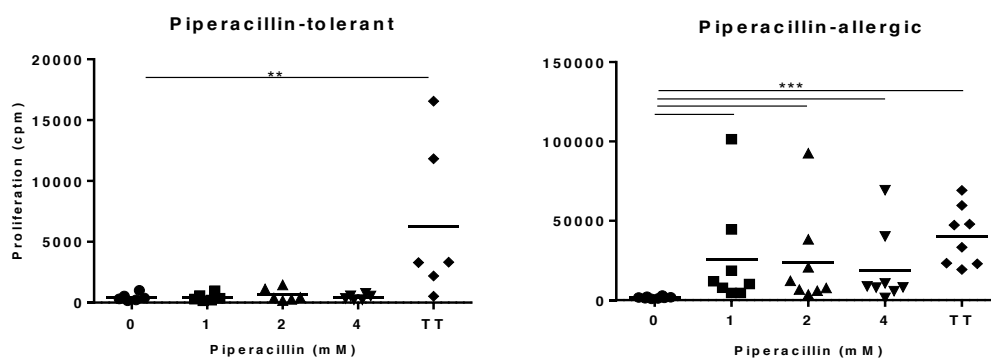


Figure 2.4 –Lymphocyte proliferation of piperacillin tolerant allergic patients. Tetanus Toxoid was used as positive control (TT= 5 µg/ml). Data statistically analysed using Mann-Whitney test accepting ** $p < 0.005$, *** $p < 0.0005$ as significant difference, control vs. treated.

2.4.2 Specificity and functionality of piperacillin responsive T-cell clones

From five piperacillin-hypersensitive patients (P1, P2, P4, P7 and P8) a total of 1420 T cell clones were generated and most of them were picked from 0.3 cell concentration per well. Of these 414 CD8+, CD4+ and CD4+8+ were stimulated to proliferate with piperacillin [Table 2.3]. CD4+8+ and CD8+ T cell clones were detected with lower frequency compared to CD4+ clones, with an approximate ratio of 1:2:4 CD4+8+, CD8+ and CD4+ T cell clones [Figure 2.5], respectively.

Patient ID	Clones tested (n)	Specific clones (n)	Proliferation (cpm±SD)		Phenotype (%)		
			Control	Piperacillin (2 mM)	CD4+	CD8+	CD4+ CD8+
P1	187	30	3232.7 ± 2566.2	10608.4 ± 10950.6	55	45	0
P2	209	120	5712.4 ± 5514.3	25709 ± 22145.9	75	13	12
P4	192	100	1361.1 ± 995.3	15526.9 ± 16868	100	0	0
P7	736	152	3954.5 ± 3721.9	12596.6 ± 11915.2	66	22	12
P8	96	12	17416.8 ± 2440.7	47903.7 ± 28946.3	N.A.	N.A.	N.A.

Table 2.3 – Origin, phenotype and specificity of piperacillin T-cell clones generated from hypersensitive patients (n= 5). Proliferative response to piperacillin responsive T-cell clones presented as [³H] thymidine incorporation (counts per minute ± SD). N.A., not analysed.

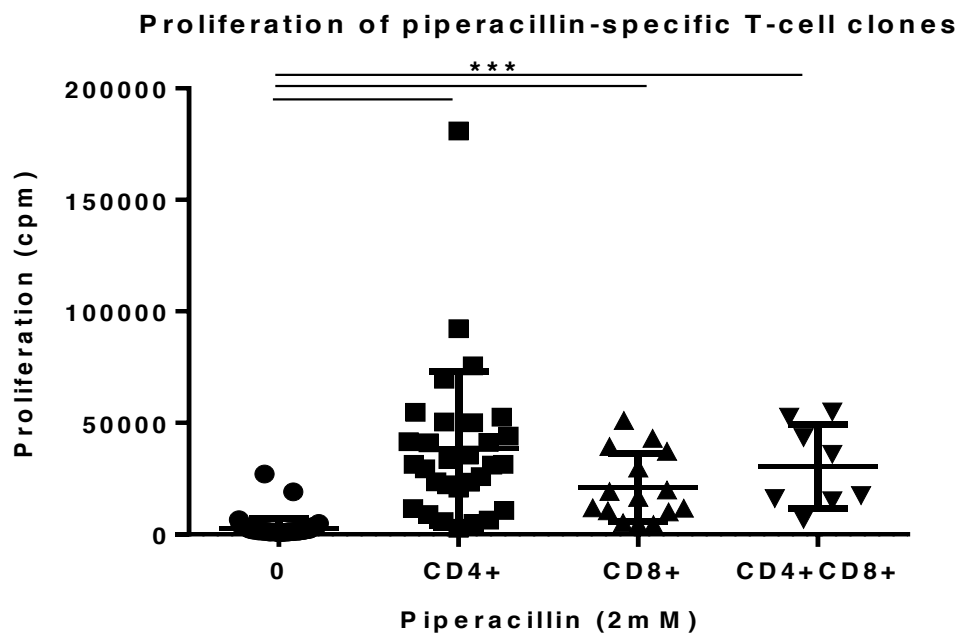


Figure 2.5 –Lymphocyte proliferation of piperacillin-specific T-cell clones. CD4+8+ and CD8+ T cell clones were detected with lower frequency compared to CD4+ clones, with an approximate ratio of 1:2:4 CD4+8+, CD8+ and CD4+ T cell clones, respectively. Data statistically analysed using Mann-Whitney test accepting ***p<0.0005 as significant difference, control vs. treated.

2.4.2.1 Piperacillin specific T-cell clones proliferate in a concentration-dependent fashion.

Out of 414 piperacillin specific T cell clones, eighty well-growing clones were chosen for further studies. These clones displayed wide-range of proliferative responses with piperacillin and expressed CD4+ and/or CD8+ receptors. The chosen T cell clones were expanded further and used in the functional studies described below to define antigen functionality and specificity.

Figure 2.6 shows 16 representative piperacillin-specific T cell clones, with different phenotypes, proliferating in response to piperacillin in a dose dependent manner when incubated with irradiated autologous EBV-transformed B-cells. In terms of drug structure, the response of CD4+, CD8+ and CD4+CD8+ T cell clones to piperacillin was found to be highly specific. Piperacillin-specific T cell clones were incubated with piperacillin and 8 structurally related compounds (1-4 mM) [Figure 1.7]. All tested clones proliferated in the presence of piperacillin, but not with the 8 structurally related compounds [Figure 2.7].

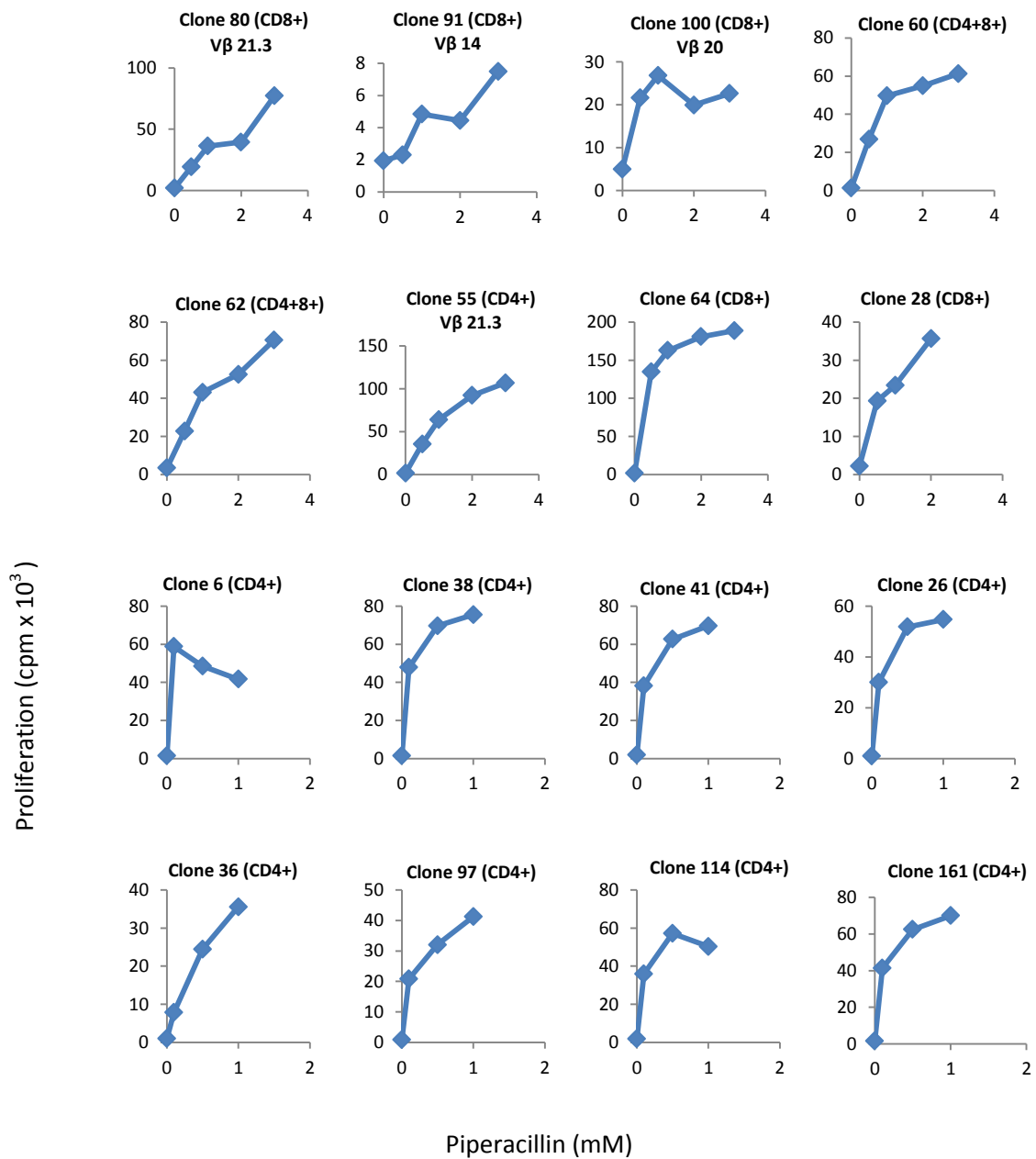


Figure 2.6 Piperacillin specific T-cell clones proliferation. Sixteen representative piperacillin-specific T-cell clones out of one hundred tested show concentration-dependent proliferation. Cells were incubated for 48h with irradiated autologous EBV transformed B-cells and piperacillin (top clones: 0.5, 1, 2 and 3 mM; bottom clones: 0.1, 0.5 and 1 mM). Data presented as average [³H]-thymidine incorporation of replicate cultures. TCR-Vβ expression is also displayed for 4 piperacillin-specific clones.

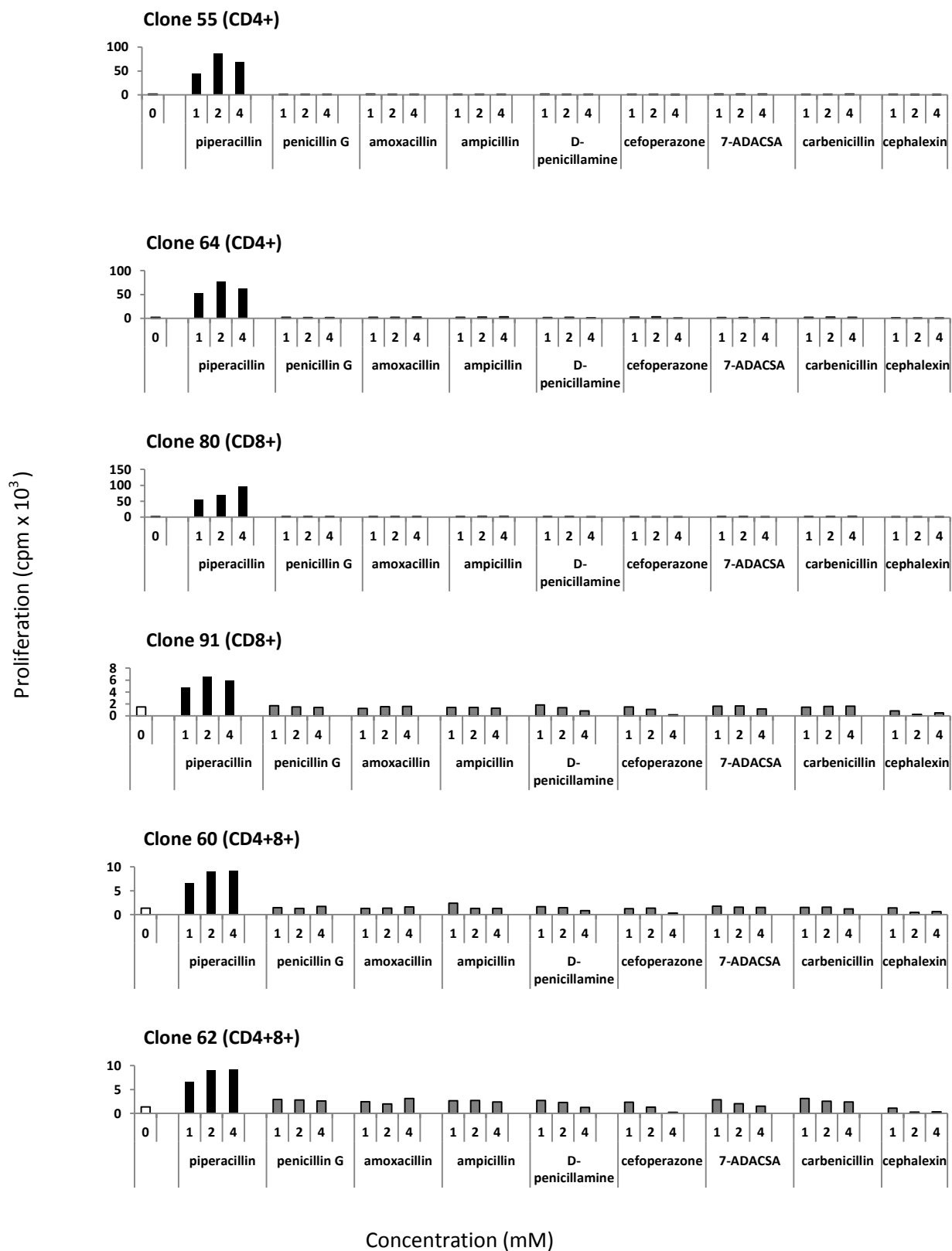


Figure 2.7- CD4+, CD8+, and CD4+8+ clones were stimulated with piperacillin, but not related drug structures. Data are presented as average cpm at each drug concentration. 7-ADACSA= 7-aminodesacetoxycephalosporanic acid. Stimulation indices were ≥ 2 with piperacillin while ≤ 2 with all other tested drugs.

2.4.2.2 Cytotoxicity of piperacillin stimulated T-cell clones

To evaluate cytotoxic activity of piperacillin-specific T cell clones a chromium-51 release assay was used. The assay involves an incubation of piperacillin-specific T cell clones for 4h with ^{51}Cr -loaded autologous EBV-transformed B-cells at different effector/target ratio (25:1 and 50:1). Piperacillin was added as an antigen in two forms: either (1) as a soluble drug (2 and 4 mM) in the traditional ^{51}Cr -release assay or (2) used to pulse antigen presenting cells (2 and 4 mM) for 16 hours prior to chromium loading in the modified ^{51}Cr -release assay. Supernatant of these cultures were collected at the end of incubation time to measure the release of radioactivity. Increased radioactivity in culture supernatant when compared with controls represents the cytolytic activity of piperacillin-specific T cell clones. Data is presented as percentage of cytolytic activity.

Cytotoxicity was undetectable with the traditional 4 hour ^{51}Cr -release assay where soluble piperacillin was used [Figure 2.8-B, Table 2.4]. However, when using the revised protocol where antigen presenting cells were pulsed with piperacillin over night are used, certain piperacillin-specific T cell clones with different phenotypes (CD4+, CD8+ and CD4+8+) displayed cytotoxic activity at an effector: target ratio of 50:1 [Figure 2.8-A, Table 2.4].

Furthermore, by using flow cytometry, we have been able to detect CD107a receptor upregulation on the surface of piperacillin-specific T cell clones after piperacillin stimulation. This indicates a cytotoxic response because normally CD107a is not detected on the cell surface unless lysosomal degranulation occurred. Increased membrane expression of the degranulation marker CD107a was detected on CD4+, CD8+ and CD4+8+ clones (n=18 out of 32 clones) [Figure 2.9].

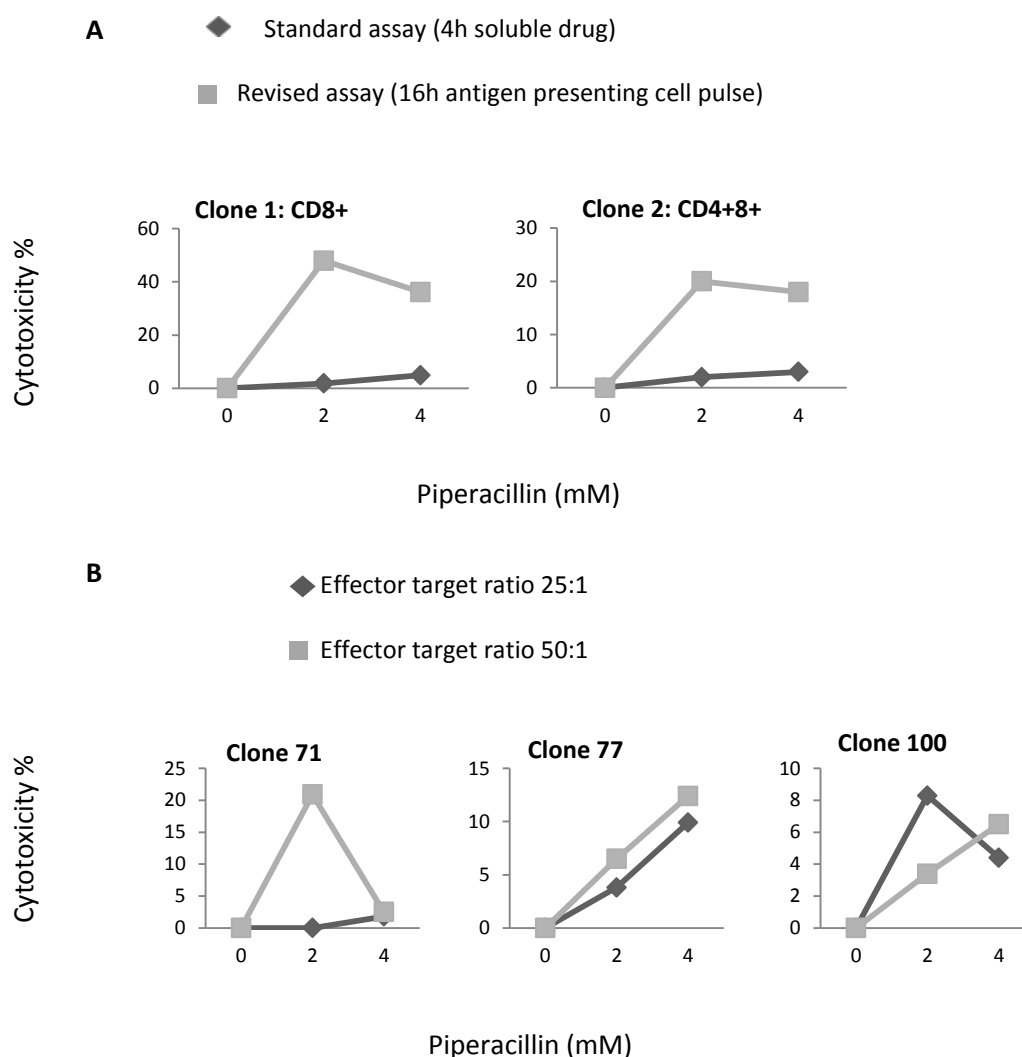


Figure 2.8- Cytotoxic activity of piperacillin specific T-cell clones. **(A)** cytolytic activity of CD8+ and CD4+8+ T-cell clones exposed to soluble piperacillin (2,4 mM) or antigen-presenting cells pulsed with piperacillin (2,4 mM) for 16h. **(B)** piperacillin specific T-cell clones were incubated with soluble piperacillin (2, 4 mM) and ^{51}Cr -loaded autologous antigen presenting cells at different effector/target ratios (25:1, 50:1). Data show the mean ^{51}Cr released from replicate wells and presented as the (%) cytotoxic activity of piperacillin treated incubations compared to controls.

Patient ID	Clone ID	Phenotype (CD)	Proliferation (SI)	Cytotoxicity %			
				Effector/target ratio			
				25:1		50:1	
				2 mM	4 mM	2 mM	4 mM
P7	Standard assay (4h soluble drug)						
	55	4	67	0	0	0	0
	64	4	110.22	2.3	2.1	4	3.6
	61	8	1.89	0	0	0.7	8.08
	66	8	N.P.	0	0	1.7	0.1
	71	8	N.P.	0	1.8	20.93	2.5
	77	8	1.6	3.8	9.9	6.5	12.4
	80	8	20.33	2.9	3.2	0	0
	91	8	2.3	0	0	0.8	3.8
	100	8	4	8.3	4.4	3.4	6.5
	105	8	N.P.	0	0	6	11.3
	60	4/8	43.5	0	0	3.3	3.25
	62	4/8	15	1.6	1.6	2.05	4.36
P4	Revised assay (16h antigen presenting cell pulse)						
	28	4	54	17.2	N.P.	20.7	N.P.
	36	4	35.6	16		33.6	
	39	4	17.9	10.9		14.4	
	74	4	29.5	9		5.2	
	18	4	40.2	11.6		N.P.	
	46	4	11.8	0		N.P.	
	58	4	32.9	N.P.		23.7	
	81	4	15.4	0		N.P.	
	92	4	4.9	7.6		N.P.	
	96	4	30.9	6.6		N.P.	

Table 2.4 Cytotoxic activity of piperacillin specific T-cell clones in response to piperacillin. T-cell clones (n= 22 clones) generated from two piperacillin hypersensitive patients [P7 (n= 12 clones), P4 (n= 10 clones)]. Data presented as percentage cytotoxicity. N.P., not performed.

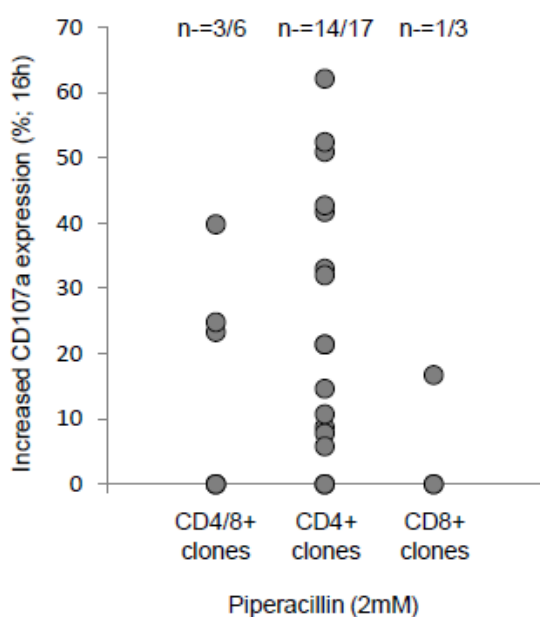


Figure 2.9- Increased CD107a expression on T-cell clones after piperacillin stimulation. Cells were labelled with anti-CD107 antibody and analysed by flow cytometry; a minimum of 30,000 cells were acquired by using forward scatter/side scatter characteristics. Results show the increase in CD107a expression when piperacillin-stimulated and unstimulated clones were compared.

2.4.2.3 Secretion of cytokines by piperacillin-specific T-cell clones.

Supernatants were collected from incubations of piperacillin-specific T cell clones (n= 7 clones) +/- piperacillin to measure cytokine release. The majority of clones secreted high levels of Th2 cytokines IL-4, IL-5 and IL-13 following piperacillin stimulation. In contrast, cytokines IFN- γ and IL-10 were secreted at lower levels after antigen stimulation. The chemokine MIP-1 β , was secreted at high levels from all piperacillin-specific T cell clones except one [Table 2.5].

Clone ID	Phenotype (CD)	Cytokine (pg/ml)								
		Proliferation (SI)	IL-4	IL-5	IL-10	IL-13	IFN- γ	TNF- α	IL-1 β	MIP-1 β
60	4/8	43.50	>10000	>10000	66	>10000	80	>10000	1	>10000
62	4/8	15.60	6974	>10000	24	>10000	3346	>10000	N.D.	>10000
64	4	110	>10000	>10000	2746	>10000	8371	>10000	2	8721
55	4	67	>10000	>10000	93	>10000	56	>10000	2	>10000
77	8	1.59	1.5	N.D.	191	3051	2404	5628	N.D.	>10000
91	8	2.30	74	1937	6	4511	370	1808	0.5	>10000
100	8	4	2740	3544	17	5897	5667	3311	N.D.	>10000

Table 2.5 Luminex analysis of secreted cytokines (pg/ml) from 7 piperacillin stimulated T-cell clones. Data represent mean of duplicate cultures with cytokine levels (less than 10 pg/ml IFN- γ ; less than 20 pg/ml IL-5, IL-1 β ; less than 50 pg/ml IL-4, IL-10, IL-13, TNF- α , MIP-1 β) in drug-free wells subtracted. N.D., not detectable.

2.5 Discussion

Piperacillin, is widely used to treat patients with CF. The preferable route to administer piperacillin is the parenteral route as it is poorly absorbed orally. 30-50% of CF patients who are treated by piperacillin suffer from mild and moderate piperacillin hypersensitive reactions (Parmar and Nasser, 2005; Burrows et al., 2007). Importantly the role of T-cells in hypersensitivity reactions in patients with CF has not been defined.

It has been shown that T lymphocytes play a vital role in coordinating immune responses against drug-derived antigen (Lopez et al., 2007; Torres et al., 2009). To activate T cells, the culprit drug should act as an antigen by ligation to specific T cell receptors. Studies by (Landsteiner and Jacobs, 1935), to break immune tolerance a drug must irreversibly bind to self-protein. Subsequently, T cells are activated by peptides liberated, by antigen processing, from the modified protein. However, Pichler and co-workers have challenged this basic concept by their influential studies. By using isolated T cells from human patients with hypersensitivity, they found that many characteristics of the drug-specific T cell response could be explained much more precisely via a reversible, direct readily interaction between the immunological receptors and the parent drug (PI concept) (Pichler, 2002a; Naisbitt et al., 2003c).

Thus, the aim of this work was to characterise the specificity and functionality of piperacillin responsive T lymphocytes in order to understand their role in non-immediate hypersensitive reactions in patients with CF.

Eight piperacillin hypersensitive patients presenting with arthritis, fever, flu-like symptoms and mild to moderate skin exanthema were included in the study. Patients' blood was collected to isolate T lymphocytes which were then used in different experimental assays to characterise and define drug-specific T cell stimulation.

Using the lymphocyte transformation test and IL-13/IFN- γ ELISpot we found drug responsive lymphocytes in all eighteen hypersensitive patients. The lymphocyte transformation test is a simple cell culture method, based on analysis of lymphocyte proliferative responses in antigen-treated and control wells. Incorporation of [3 H]-thymidine is used to measure levels of proliferation. The lymphocyte transformation test has been used extensively in specialised facilities for the diagnosis of non-immediate cutaneous hypersensitivity reactions. Detailed review (Nyfeler and Pichler, 1997; Pichler and Tilch, 2004) of the published literature revealed that the assay has an estimated sensitivity of 78% (the percentage of positive results in hypersensitive patients) and specificity (percentage of responders in control groups) approaching 85%. In our laboratory similar results have been obtained in patients hypersensitive to the anti-convulsants carbamazepine and lamotrigine (Naisbitt et al., 2003a; Naisbitt et al., 2003b; Wu et al., 2006; Wu et al., 2007). ELISpot assays involve similar culture conditions, i.e., suspect drug antigens are titrated in PBMC cultures; however, the end-point to detect drug-specific T-cell proliferation is cytokine secretion. A detailed comparison of lymphocyte proliferative responses and cytokine secretion in penicillin hypersensitive patients indicated that IFN- γ ELISpot was the most sensitive and could be used in a greater number of individuals for the diagnosis of penicillin hypersensitivity. Our study found similar results in that piperacillin-specific IFN- γ secretion was detected in all 8 hypersensitive patients. Analysis of the Th2 cytokine IL-13 revealed that a portion of hypersensitive patients PBMC secrete a mixed panel of cytokines (discussed in more detail below).

The proliferative response of piperacillin-specific T lymphocytes was dose-dependent and reproducible on repeated testing, with the maximum response noticed within therapeutic piperacillin concentrations that are likely to be in the range of 0.5-1 mM. These data indicate that T-cells likely participate in the cutaneous manifestations of piperacillin hypersensitivity. It is also possible that drug-specific lymphocytes are also involved in other clinical features of the disease (flu-like symptoms, arthralgia and fever). This conclusion is based on the fact that

piperacillin-specific lymphocytes were detected in both hypersensitive patients with and without cutaneous symptoms.

Importantly, the lymphocyte transformation test was negative in all five tolerant controls; thus, in our limited control the lymphocyte transformation test specificity was 100%. Accordingly, in regard with the diagnosis of piperacillin allergy, the *in vitro* assay revealed an advantage over the skin testing (Pichler and Tilch, 2004).

The nature of the induced immune response is determined by the cytokine profile accompanied with a particular reaction (Hertl et al., 1993a; Hertl et al., 1993b; Yawalkar et al., 2000; Beeler et al., 2006; Lochmatter et al., 2009; Rozieres et al., 2009). A mixed panel of cytokines (IL-13, IFN- γ , IL-6, IL-1 β , TNF- α , and MIP-1 α/β) was detected after antigen stimulation of piperacillin hypersensitive patients lymphocytes. IL-6, IL-1 β and TNF- α detection in the supernatant of piperacillin containing cultures are indicative of a dendritic cell response against piperacillin, which agrees with what has been recently described with amoxicillin (Rodriguez-Pena et al., 2006).

Over four hundred piperacillin-specific T cell clones were generated from peripheral blood of five patients with piperacillin hypersensitivity. The most dominant phenotype of the clones was the CD4+. All different T-cell phenotypes (CD4+, CD8+ and CD4+CD8+) were detected, indicating that both CD4+ and CD8+ T-cells participate in the disease pathogenesis [Figure 2.5]. High levels of TNF- α , MIP-1 β and Th2 cytokines (IL-4, IL-5 and IL-13) was secreted from T cell clones after piperacillin stimulation [Table 2.5]. IL-10 and IFN- γ were also secreted by all stimulated clones with piperacillin but in low levels, which probably links to the positive IFN- γ ELISpot detected using peripheral blood lymphocytes. The different profile of cytokines seen in PBMC and clone assays may relate to the methods employed (PBMC, ELISpot, clone, Luminex). It might however relate to the uncovering of T-cell populations that are masked in PBMC cultures, which is an area of on-going research in our laboratory.

Cytolytic activity was detected against autologous antigen presenting cells pulsed with piperacillin, using a revised ^{51}Cr - release assay which accounts for the time required for piperacillin to modify protein and form a T-cell antigen. Moreover, up-regulation of CD107a, a lysosomal membrane marker, was detected on the surface of CD4+, CD8+ and CD4+CD8+ clones after piperacillin stimulation demonstrating their degranulation and cytolytic activity. These data indicate that piperacillin-specific clones' have the potential to cause tissue injury and the adverse reactions seen in patients with CF.

β -Lactam antibiotics are known to be targeted by specific Lys residues on protein (Levine and Ovary, 1961; Batchelor et al., 1965b; Meng et al., 2011). Nucleophilic attack leads to ring opening, binding of the penicilloyl group, and ultimately formation of a stable adduct. We found that clones were stimulated to proliferate with piperacillin, but not other β -lactam-containing antibiotics. This indicates that the penicilloyl core structure alone does not deliver specific antigenic signals to activate T-cells. To investigate the fine specificity of the piperacillin-specific T-cell response, clones were cultured with cefoperazone, which contains a side chain closely related in structure to piperacillin, whereas the thiazolidine ring is replaced by a six-membered ring. Once more, clones were activated with piperacillin, but not with cefoperazone, demonstrating that piperacillin-responsive T-cells are highly drug-specific and recognise the penicilloyl structure and the specific side chain of piperacillin.

To conclude, by utilising blood samples from hypersensitive patients with CF we have been able to characterise antigen-specific CD4+,CD8+ and CD4+8+ T cells. The existence of these cells together with their cytolytic features suggests that they may play a role in the pathogenesis of the disease.

Chapter Three

The role of T-lymphocytes in cystic fibrosis patients with multiple beta-lactams hypersensitivity

3.1 Introduction	88
3.2 Aims	89
3.3 Patients and methods	89
3.3.1 Patients' demographic	89
3.4 Results	91
3.4.1 Stimulation of PBMC from hypersensitive patients to piperacillin, aztreonam and meropenem	91
3.4.2 Generation of piperacillin, aztreonam and meropenem-responsive T cell clones	93
3.4.3 Drug-antigen specificity of T cell clones	94
3.5 Discussion	97

3.1 Introduction

β -lactam antibiotics provide the foundation for treatment of pulmonary infections in patients with CF. Unfortunately, the use is often limited by a high frequency of hypersensitive reactions. Several studies report a reaction prevalence of 26-50%, compared with 1-10% in the general population (Pleasant et al., 1994; Parmar and Nasser, 2005; Burrows et al., 2007; Whitaker et al., 2011b). β -lactam allergies in patients with cystic fibrosis are usually non-immediate with a mean time of onset of 9.1 days (Whitaker et al., 2011a). Reactions consist of rashes, fever and/or flu-like symptoms. In 375 patients at the Leeds Regional Adult Cystic Fibrosis Unit only 6% of 302 β -lactam reactions were identified as being immediate. Severe skin reactions and systemic involvement were not encountered.

To explore the immunological mechanisms of β -lactam allergy in patients with cystic fibrosis, we have previously focused on piperacillin. Peripheral blood mononuclear cells (PBMC) isolated from 68% of allergic patients, but not drug-exposed tolerant controls, were found to proliferate following piperacillin stimulation *in vitro* (Whitaker et al., 2011a). In chapter 3, over 400 piperacillin-responsive T-cell clones were isolated from hypersensitive patients and used to characterize the cellular pathophysiology of the reaction. Drug-responsive clones were CD4⁺ and CD8⁺ and secreted IFN- γ , TNF- α and Th2 cytokines.

If an allergic reaction to a β -lactam antibiotic such as piperacillin (penicillin class) is observed, it is replaced by another drug often from a different class (e.g., aztreonam from a monobactam class, meropenem from carbapenem class) [Figure 1.6]. However, approximately 20% of patients develop allergic reactions to these drugs (Whitaker et al., 2011b). Patients who have exhausted all conventional therapeutic options are usually older and sicker and require more frequent treatment. This results in prolonged hospital admissions for observation of treatment.

Clinical studies suggest that the extent of cross-reactivity between different classes of β -lactam antibiotic is low. Moss et al. (Moss et al., 1991) reported that 19 out of 20 patients with previous penicillin or cephalosporin allergy safely tolerated aztreonam. Cross-reactivity rates between penicillin and meropenem in patients with immediate reactions are estimated at less than 1%; however, several patients have been reported to become sensitized against the alternative drug following repeated exposure (Moss, 1991; Moss et al., 1991; Atanaskovic-Markovic et al., 2008; Frumin and Gallagher, 2009).

3.2 Aims

The factors that drive allergic reactions against multiple drugs are not known. We therefore looked to:

- Identify drug-responsive PBMC in multi-allergic patients with cystic fibrosis.
- Generate drug-responsive T-cell clones to investigate T-cell receptor cross-reactivity with the different β -lactam antibiotics.

3.3 Patients and methods

3.3.1 Patients' demographics

Four patients with cystic fibrosis and allergies to piperacillin, aztreonam and meropenem, and four drug-exposed tolerant patients with cystic fibrosis were recruited. Table 3.1 summarises the patients' demographics and the clinical features of the reactions. Approval for the study was acquired from the local research ethics committee, and informed written consent was obtained from each donor. Detailed experimental method for cloning, specificity and ELISpot assays are described in chapter two.

Allergic patients							
ID	Age/sex	Drug reaction	Details of the reaction	Time to reaction (days)	Time since reaction (years)	Courses prior to reaction	LTT ^a (maximum SI)
1	21/M	Piperacillin	MPE ^b , fever	9	7	3	13.3
		Aztreonam	MPE	4	5	2	<2
		Meropenem	MPE	5	5	3	8.0
2	26/M	Piperacillin	MPE	5	2	3	31.1
		Aztreonam	Urticarial rash	2	1	4	2.4
		Meropenem	MPE	3	3	5	4.4
3	28/F	Piperacillin	Fever, arthralgia	9	6	7	6.8
		Aztreonam	Fever, arthralgia	6	5	4	4.1
		Meropenem	Flu-like symptoms, vomiting	6	4	5	5.8
4	22/M	Piperacillin	MPE/fever	2	1	4	62.5
		Aztreonam	Urticarial rash	1	1	2	<2
		Meropenem	Lip swelling	1	2	3	4
Tolerant patients							
ID	Age/sex	Drug reaction	Drug exposure				LTT (maximum SI)
1	28/M	none	exposed to piperacillin, aztreonam and meropenem in the last 3 years In 1 year prior to his death, he required 73 days of antibiotic treatment.				<2 ^c
2	24/F	none	exposed to piperacillin, aztreonam and meropenem in the last 3 years In the last 12 months, the patient received 33 days of antibiotic treatment.				<2
3	21/M	none	exposed to piperacillin, aztreonam and meropenem in the last 3 years averaged 81 days of antibiotic treatment per year				<2
4	29/M	none	exposed to piperacillin, aztreonam and meropenem in the last 3 years averaged 14 days of antibiotic treatment per year				<2

Table 3.1 – Clinical details of the tolerant and allergic patients. ^a LTT, lymphocyte transformation test; ^b MPE, maculopapular eruption; ^c <2, LTT SI <2 with each drug (0.1-2mM).

3.4 Results

3.4.1 Stimulation of PBMC from allergic patients with piperacillin, aztreonam and meropenem

PBMC from all four multi-allergic patients were stimulated to proliferate in the presence of piperacillin, aztreonam and meropenem [Table 3.1]. The proliferative response was drug concentration dependent and associated with the release of IFN- γ and/or IL-13 [Figure 3.1]. In contrast, PBMC from patients exposed to several courses of the three drugs with no apparent allergies were not stimulated to proliferate or release cytokines [Table 3.1].

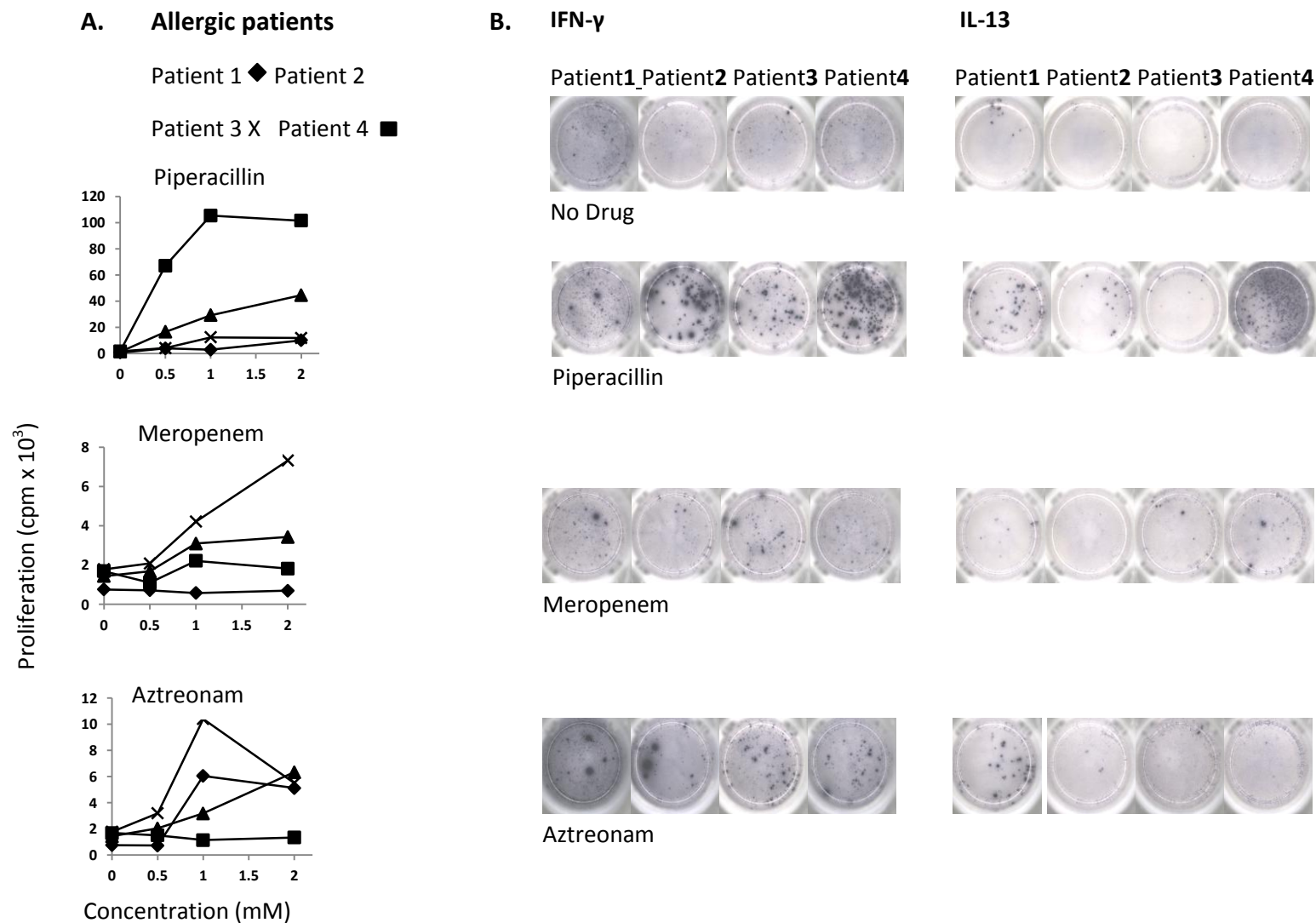


Figure 3.1- Drug-specific stimulation of allergic patients PBMC. (A) PBMC were cultured for 5 days in the presence of piperacillin, meropenem, or aztreonam. Proliferation was measured on day 6 by the addition of [3H]-thymidine for the last 16h of the incubation. Results are shown as the mean cpm readings of triplicate cultures. (B) Drug-specific ELISpot for IFN- γ and IL-13. (0.5×10^6) PBMC from allergic patients secreted IFN- γ and IL-13 after 48h of incubation with drugs. Well images were taken by AID Software.

3.4.2 Generation of piperacillin, aztreonam and meropenem-responsive T cell clones

One thousand six hundred and sixty three T-cell clones were generated from the 4 allergic patients following PBMC stimulation with piperacillin, aztreonam or meropenem and tested for drug specificity. A total of 122 were found to display reactivity against piperacillin (n=89), aztreonam (n=22) or meropenem (n=14) [Figure 3.2]. Although the majority of drug-response clones were CD4+, CD8+ clones were generated from each allergic patient. The number of piperacillin-, aztreonam and meropenem-responsive clones generated from each patient and the phenotype is summarised in Table 3.2. Sixty well-growing CD4+ and CD8+ clones selected from all 4 allergic patients were used to characterise concentration-dependent proliferative responses, cytokine release and cross-reactivity.

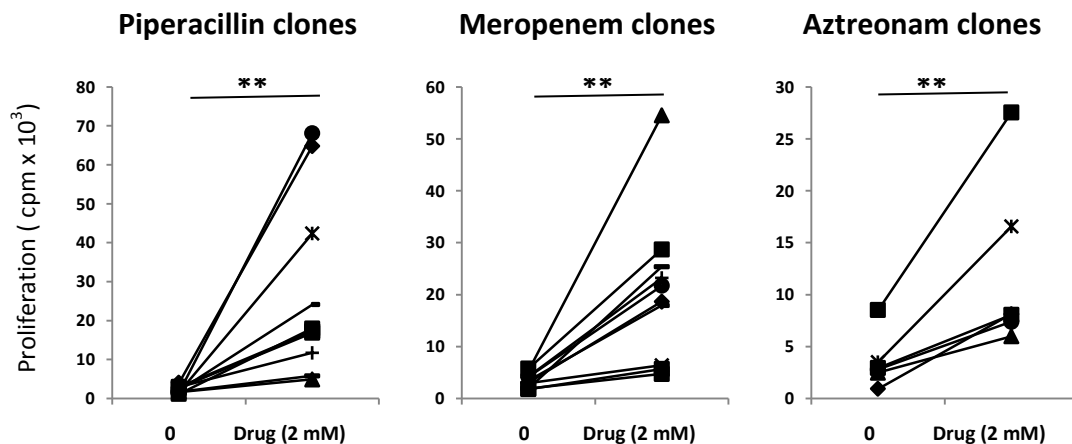


Figure 3.2- T-cell clones activation with piperacillin, meropenem and aztreonam. T-cell clones were cultured for 72h with antigen presenting cells and the drugs (2 mM). The figure displays the proliferative response of representative clones, piperacillin (n = 10), meropenem (n = 6) and aztreonam (n = 10). Data statistically analysed using Student's *t*-test accepting ***p*<0.005 as significant difference, control vs. treated

ID	Drug	Tested clones (n)	Specific clones (n)	Proliferation (cpm)		Phenotype (%)		
				control	Drug (2 mM)	CD4+	CD8+	CD4+CD8+
1	Piperacillin	58	2	1422 ± 365	10512 ± 7907	100		
	Aztreonam	111	2	2043 ± 788	6683 ± 2042	100		
	Meropenem	111	5	4340 ± 3988	11744 ± 11173	80	20	
2	Piperacillin	67	23	5477 ± 4137	36004 ± 23732	78	9	13
	Aztreonam	160	6	3529 ± 1761	15711 ± 19623	65	17	17
	Meropenem	100	4	1046 ± 441	2399 ± 938	100		
3	Piperacillin	144	16	8133 ± 4353	53954 ± 18612	50	50	
	Aztreonam	216	13	8984 ± 5963	23323 ± 12356	84	8	8
	Meropenem	72	1	3504	9550	100		
4	Piperacillin	216	48	2134 ± 2412	24461 ± 18542	88	6	6
	Aztreonam	208	1	6867	33942	100		
	Meropenem	200	5	3795 ± 2827	11493 ± 6948	100		

Table 3.2- Origin, specificity and phenotype of T-cell clones from allergic patients.

3.4.3 Drug-antigen specificity of T cell clones

Proliferation of piperacillin-, aztreonam- and meropenem-responsive CD4+ and CD8+ clones was drug concentration-dependent; maximal responses were detected between 0.5-2mM [Figure 3.3]. Around one thousands and six hundred sixty three clones were generated but only one hundred and twenty six were drug-specific. Fifty five clones were tested for reactivity against piperacillin, aztreonam and meropenem (figure 3.4 showed only six representative clones) and the response to each drug was found to be highly specific. Clones were stimulated to proliferate [Figure 3.3] and release IFN- γ [Figure 3.4] in the presence of the drug PBMC were cultured with to generate the clones, but not the other β -lactam antibiotics.

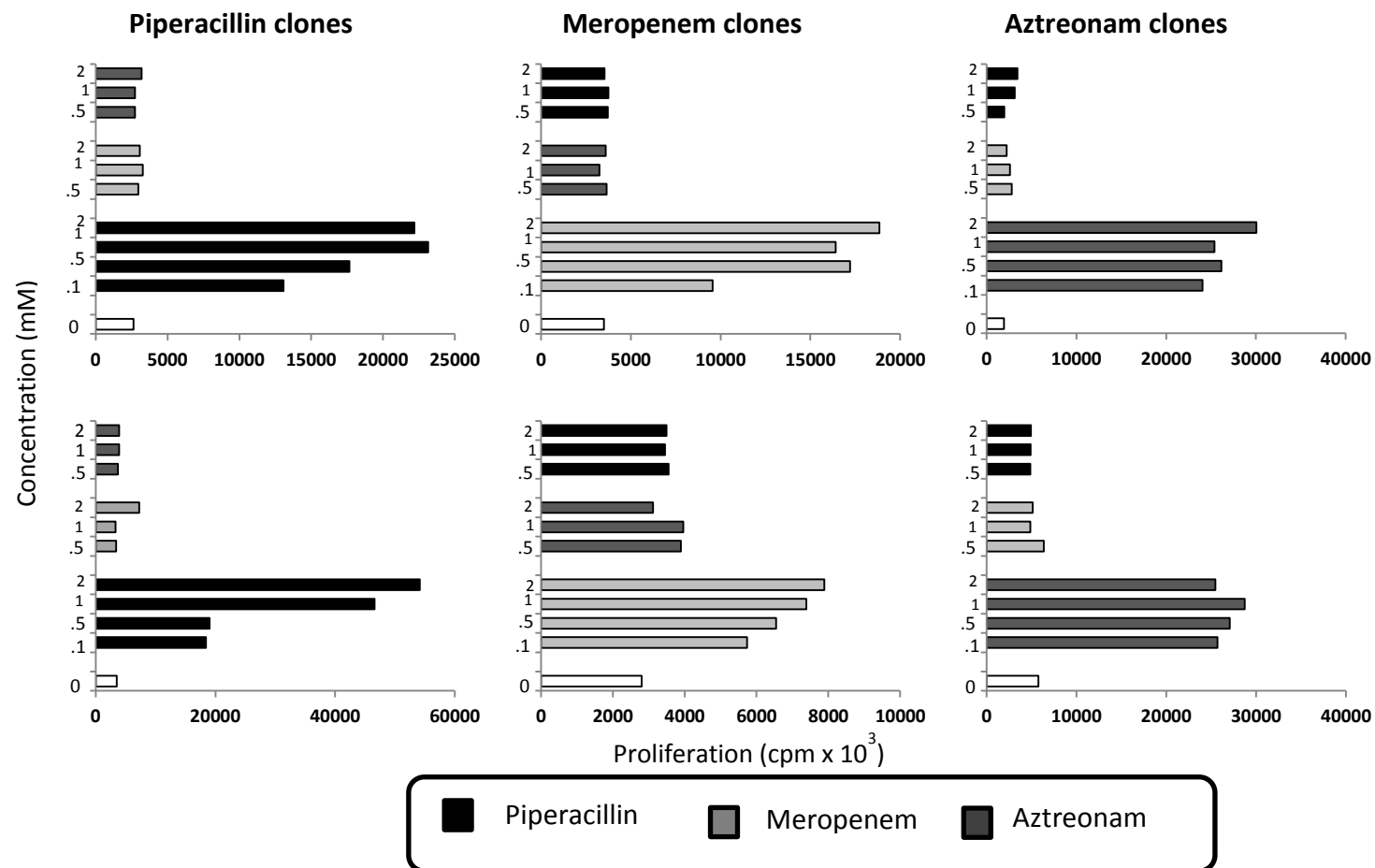


Figure 3.3 -Cross reactivity of piperacillin, meropenem, and aztreonam T cell clones. Clones were incubated for 72h with antigen presenting cells in the presence of the drugs (0.1-2 mM). Proliferative responses were measured by [³H]-thymidine incorporation of the final 16h of the incubation. Results are shown as the mean cpm of triplicate cultures.

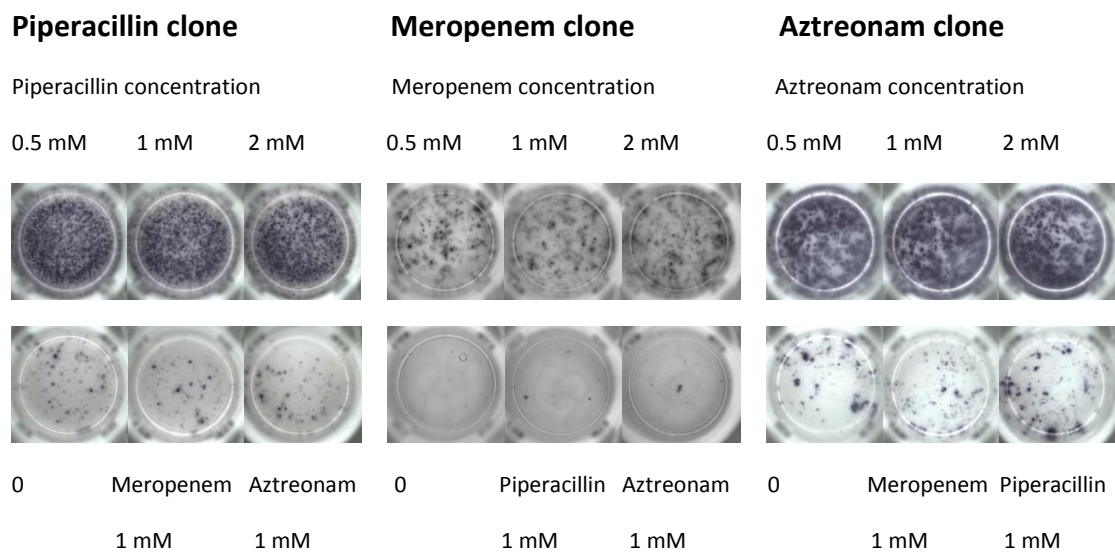


Figure 3.4- Drug-specific IFN- γ ELISpot. T cell clones generated from patients were stimulated with the drugs for 48h. Each drug-specific clone was tested with the other 2 drugs to test its cross-reactivity. Well images were taken by AID Software.

3.5 Discussion

β -lactam antibiotics form covalent bonds with lysine residues on protein in drug exposed patients (Levine, 1960; Batchelor et al., 1965b; Jenkins et al., 2009a; Whitaker et al., 2011a) to generate antigenic determinants for T-cells. Several studies have shown that processing of β -lactam albumin conjugates liberate peptide sequences that stimulate T-cells (Brander et al., 1995; Padovan et al., 1997; Whitaker et al., 2011a; El-Ghaiesh et al., 2012). Under in vitro conditions, β -lactams bypass this requirement for protein processing by binding directly to MHC-associated peptides (Brander et al., 1995; Padovan et al., 1996a). Cross-reactivity studies using PBMC and T-cell clones from penicillin allergic patients reveal a plethora of drug response profiles (Mauri-Hellweg et al., 1996; Sachs et al., 2004; Rozieres et al., 2009). In most allergic patients, the peptide-bound penicilloyl ring structure is thought to represent the main structural determinant for T-cell receptors, as β -lactams that contain a different core structure do not stimulate T-cells. However, individual T-cells display a range of side-chain reactivity. Cross-reactive clones can be stimulated with several penicillins, some of which the allergic patient will have never knowingly been exposed to. In contrast, other clones are incredibly drug antigen-specific. Our recent studies with piperacillin mono-allergic patients revealed that PBMC and clones were not stimulated with other penicillins (see chapter 2). Collectively, these data suggest that different antigenic determinants derived from penicillins can be accommodated within the MHC binding cleft without interfering with the T-cell receptor binding interaction. However, the degree of cross-reactivity observed is drug and patient-specific and might be influenced by previous drug exposure. Thus, there is a need to further delineate the structural features that determine T-cell receptor cross-reactivity.

Symptoms of the reactions were classified according to patient's medical records. Patients 1, 2 and 4 developed maculopapular exanthema and/or non-immediate urticarial eruptions against the 3 drugs [Table 3.1]. Patient 3 developed less-classical symptoms of drug allergy (arthralgia, nausea and vomiting). Reactions were relatively mild but in every case treatment had to be discontinued. Each patient received and tolerated several courses of the individual drugs prior to the allergic

reaction. Our primary objective was to determine whether it was possible to detect T-lymphocyte responses to each drug in the allergic patients. A secondary objective was the analysis of T-cell receptor cross-reactivity through the generation of T-cell clones.

The lymphocyte transformation test and IFN- γ ELISpot are the biological assays most commonly applied to diagnose drug allergy in vitro (Pichler and Tilch, 2004; Rozieres et al., 2009). Our study utilised both approaches to show that piperacillin, aztreonam and meropenem stimulate PBMC from allergic patients, but not drug-exposed tolerant controls, to proliferate and secrete cytokines. These data confirm our previous findings in chapter 2 with piperacillin hypersensitive patients and demonstrate that T-cell responses are also readily detectable with aztreonam and meropenem. The drug-specific response was dose-dependent and reproducible on repeated testing. PBMC were stimulated with similar concentrations of the drugs. For three of the patients piperacillin stimulated the strongest proliferative response and the highest levels of IFN- γ secretion, when the three drugs were compared [Table 3.1; Figure 3.1]. This may relate to intrinsic chemical reactivity and differences in the level of drug protein conjugates formed in vitro.

CD4⁺, CD8⁺ and CD4⁺CD8⁺ T-cell clones were isolated from piperacillin, aztreonam and meropenem-stimulated PBMC to explore T-cell receptor cross-reactivity. Antigen-specificity was measured using proliferation and cytokine release as readouts and no cross-reactivity was observed. Over 50 clones were analysed [Figure 3.2]. These data clearly demonstrate that non-immediate reactions to different classes of β -lactam antibiotic in patients with cystic fibrosis are instigated, not by cross-reactive T-cells, but through priming naive T-cells against the different drug antigens. Daubner et al. (Daubner et al., 2012) have recently demonstrated that drug-responsive T-cells in certain patients with a history of multiple drug allergies reside in an in vivo activated (CD4⁺CD25^{dim}, with elevated CD38 and PD-1) T-cell fraction. Thus, we are currently exploring whether a similar phenotype of drug-responsive T-cells exist in patients with cystic fibrosis and multiple drug allergies

and indeed whether increased numbers of such T-cells might explain why multiple drug allergy is such a common occurrence in this patient group.

Chapter Four

Characterisation of flucloxacillin specific T-lymphocyte responses in drug-induced liver injury

4.1 Introduction	101
4.2 Aims	102
4.3 Patients and methods	102
4.3.1 Patients' demographics	102
4.3.2 Detection of flucloxacillin-specific PBMC responses	104
4.3.3 Generation of T-cell clones from patients with flucloxacillin-induced liver injury	104
4.3.4 Protocol for priming naive T Cells from healthy volunteers with flucloxacillin	104
4.3.5 Phenotype and specificity of T-cell clones	106
4.3.6 Migration assay	106
4.3.7 Mechanistic studies of antigen presentation	107
4.4 Results	107
4.4.1 Flucloxacillin-specific PBMC responses in patients with DILI	107
4.4.2 Characterisation of flucloxacillin-responsive CD4+ and CD8+ T-cell clones from patients with DILI	109
4.4.3 Flucloxacillin activates naïve CD45RA+ CD8+ T cells from volunteers expressing HLA-B*57:01	112
4.4.4 Flucloxacillin-responsive CD8+ T cells are restricted by HLA-B*57:01 and HLA-B*58:01	114
4.4.5 Expression of distinct patterns of homing receptors on flucloxacillin-specific T-cell clones	118
4.5 Discussion	120

4.1 Introduction

Adverse drug reactions are a major complication of drug therapy and an impediment to drug development. Immunological reactions are extremely important because of their severity and they account for many cases of drug withdrawal. They cannot easily be predicted and no simple dose-response is discernible. Skin is the tissue most commonly targeted by immune cells; however, other organs, including the liver, can be damaged either in isolation or as part of a generalised hypersensitivity syndrome (Park et al., 2011).

T-lymphocytes are believed to cause drug-induced skin injury through the action of cytokines and cytolytic molecules. To stimulate a T-cell response the drug must bind to human leukocyte antigen (HLA) and in some way crosslink specific T-cell receptors. Recently, a number of cutaneous drug reactions have been strongly associated with expression of HLA alleles (e.g., abacavir hypersensitivity [HLA-B*57:01] (Mallal et al., 2002), carbamazepine hypersensitivity in Caucasians, and Japanese [HLA-A*31:01] (McCormack et al., 2011; Ozeki et al., 2011) carbamazepine-induced Stevens Johnson syndrome in Han Chinese [HLA-B*15:02]) (Chung et al., 2004), which implies a direct/indirect effect of the gene product on the disease. For abacavir and carbamazepine, it has been possible to relate the genetic association to the mechanism of disease by characterising drug-specific CD8⁺ T-cell responses in volunteers expressing HLA-B*57:01 and B*15:02, respectively (Chessman et al., 2008; Ko et al., 2011).

The role of T cells in drug reactions targeting the liver is less well defined. In 1997, Maria and Victorino (Maria and Victorino, 1997) described lymphocyte proliferative responses to drugs in over 50% of patients with drug-induced liver injury (DILI). More recently, histological examination of an inflamed liver from a single patient exposed to sulfasalazine revealed an infiltration of granzyme B secreting T-lymphocytes (Mennicke et al., 2009). The discovery of HLA alleles as risk factors for DILI (e.g., flucloxacillin [B*57:01] (Daly et al., 2009), ximelagatran [DRB1*07:01] (Kindmark et al., 2007), lumiracoxib [DRB1*15:01] (Singer et al.,

2010)) is supportive of an immune mechanism; however, biological data showing HLA restriction of drug-responsive cytotoxic T cells is lacking.

4.2 Aims

The strength of the association described for flucloxacillin—approximately 85% of cases carry at least one copy of HLA-B*57:01—prompted us

- To investigate the cellular response in patients with flucloxacillin-induced liver injury.
- To investigate whether flucloxacillin activates naïve CD8⁺ T cells from HLA-B*57:01 positive volunteers.

4.3 Patients and Methods

4.3.1 Patients' demographics

Six patients with flucloxacillin-induced liver injury and six flucloxacillin-exposed tolerant controls were recruited. Table 4.1 lists the clinical features of the reactions and concomitant medications. Three HLA-B*57:01-positive flucloxacillin-naïve individuals were also selected from our frozen cell bank containing peripheral blood mononuclear cells (PBMCs) from four hundreds healthy volunteers recruited from northwest England. A total of 100 mL of blood was collected for both DNA and PBMC isolation. Genomic DNA was extracted using Chemagic magnetic separation (Chemagen, Baesweiler, Germany) and high-resolution sequence-based HLA typing was performed by the Histogenetics laboratory (Histogenetics, Ossining, NY) at the following loci: HLA-A, -B, -C, -DRB1, -DQB1, and DQA1. Approval for the study was acquired from the Liverpool local Research Ethics Committee and informed written consent was obtained from each donor.

Subject ID	Age	sex	Peak liver function tests at time of liver injury*				Time to onset	Since reaction	International Consensus Criterion RUCAM _‡ score	Concomitant medication and comments
			ALT	Bilirubin	ALP	GGT	(weeks)	(Years)	3-5 Possible, 6-8 Probable, >8 Highly probable	
P1	73	F	18x ULN	7x ULN	6x ULN	22x ULN	4	11	10	Reaction included maculopapular exanthema. Other causes of cholestatic hepatitis excluded
P2	61	M	23x ULN	3x ULN	9x ULN	Not measured	3	11	7	Eosinophilia and arthralgia; Other causes of cholestatic hepatitis excluded
P3	73	F	13x ULN	12x ULN	2x ULN	12x ULN	4	2	3	Also taking co-amoxiclav at time of the reaction. Other causes of cholestatic hepatitis excluded
P4	90	F	11x ULN	13x ULN	4.5x ULN	14x ULN	3	3	6	Other causes of cholestatic hepatitis excluded
P5	65	F	4x ULN	36x ULN	2x ULN	1.5x ULN	3	3	5	Also taking co-amoxiclav at time of the reaction. Reaction included maculopapular rash. Other causes of cholestatic hepatitis excluded
P6	78	F	36x ULN	17x ULN	5x ULN	16x ULN	3	6	10	Reaction included maculopapular exanthema. Other causes of cholestatic hepatitis excluded

Table 4.1 Clinical features of the patients. *ULN, upper limit of normal; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase. ‡ The cases were evaluated by application of the Council for International Organisations of Medical Science scale, also called the Roussel Uclaf Causality Assessment Method (RUCAM). The pattern of liver injury was classified according to the International Consensus Meeting Criteria. Only cases having at least possible causality (score 3+) were included in the study. Diagnosis of DILI was done by expert hepatologists.

4.3.2 Detection of flucloxacillin-specific PBMC responses

Proliferation of patient PBMC (0.15×10^6 /well) against flucloxacillin (0.1-2 mM) and tetanus toxoid ($5 \mu\text{g/mL}$) was measured using the lymphocyte transformation test. Interferon-gamma (IFN- γ) and granzyme B-secreting PBMCs were visualized using ELISpot (MabTech, Nacka Strand, Sweden) by culturing PBMC (0.5×10^6 /well; $200 \mu\text{L}$) with flucloxacillin (1–2 mM) or PHA ($5 \mu\text{g/mL}$) for 48 hours. Detailed experimental procedure is described in chapter 2.

4.3.3 Generation of T-cell clones from patients with flucloxacillin-induced liver injury

PBMC (1×10^6 /well; 0.5 mL) from patients with DILI were cultured with flucloxacillin (1-2 mM) in RPMI 1640 supplemented with 10% human AB serum (Innovative Research, Class A), 25 mM HEPES, 10 mM L-glutamine, and $25 \mu\text{g/mL}$ transferrin (Sigma-Aldrich, Gillingham, UK). Cultures were supplemented with 200 IU/mL rhIL-2 (PeproTech, London, UK) on days 6 and 9. On day 14, CD8 $^{+}$ cells were isolated by positive selection using CD14 microbeads (Miltenyi Biotec, Bisley, UK) and the remaining cells designated as CD4 $^{+}$. The separated cells were then cloned by serial dilution (see chapter 2).

Epstein-Barr virus (EBV) transformed B-cell lines were created from PBMC by transformation with supernatant from the virus-producing cell line B9.58. Lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Invitrogen, Paisley, UK), 100 mM L-glutamine, $100 \mu\text{g/mL}$ penicillin, 100 U/mL streptomycin, and used as a source of autologous antigen-presenting cells.

4.3.4 Protocol for priming naive T Cells from healthy volunteers with flucloxacillin

T-cell priming was performed with naive CD3 $^{+}$ T cells using our recently established protocol (Faulkner et al., 2012b). Blood was taken from healthy volunteers and PBMCs were isolated following previously described protocol.

CD14⁺ cells were isolated by positive selection utilising CD14 microbeads. CD4 naïve T-cells were isolated by negative selection which involves CD25 depletion. CD3 cells were also isolated by negative selection utilising the Pan T isolation kit II, then CD25 and CD45RO positive cells were removed by positive selection leaving untouched CD3 naïve cells. All cell isolations were carried out according to manufacturer's instructions (Miltenyi Biotec Ltd). Isolated cells were phenotyped by flow cytometry using CD25-PE, CD8-FITC, CD4-PE, CD45RA-FITC, CD45RO-PerCP-Cy5.5, CD3-APC and CD14-FITC antibodies. The purity of isolated naïve T-cells was more than 97%.

Isolated CD14⁺ monocytes were differentiated for 7-8 days in medium containing 800 U/ml IL-4 and 800 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) (Pepro Tech EC Ltd., London, U.K.) to generate dendritic cells. Either fresh or frozen cells were resuspended at $1-2 \times 10^6$ cells/ml and seeded at 3ml/well in six-well culture plates. Every 2 days, cells were fed with 3ml/well fresh medium containing 800 U/ml IL-4 and 800 U/ml GM-CSF. Tumour necrosis factor alpha (TNF- α); 25ng/ml (PeproTech) and lipopolysaccharide (LPS); 1 μ g/ml (E. coli strain 0111:B4, Sigma-Aldrich) were added for the last 16 hours of culture. The phenotype of immature Monocyte derived dendritic cells (Mo-DC) was detected by flow cytometry. Cells were CD1a and CD14negative, and CD11a, CD11c, CD40, CD80, CD83, CD86, CD274 and HLA Class II positive.

At the end of 7-8 days incubation, immature Mo-DC were harvested and seeded at 8×10^4 cell/well in 500 μ l in 24-well culture plates. Naïve CD3 T-cells were thawed quickly at 37°C then washed and added at 2×10^6 cell/well in 1ml of fresh medium. Flucloxacillin was then added at 1-2mM in 500 μ l to cells. Cultures were incubated at 37°C in 5% CO₂ for 7-8 days. Nitroso sulfamethoxazole (50 μ M) was used as a control antigen in all experiments.

After the co-culture period, primed T cells (1×10^5 /well; 200 μ L) were restimulated with flucloxacillin (0.5-2 mM) or nitroso sulfamethoxazole (10-80 μ M), and fresh

dendritic cells (4×10^3 /well). Proliferation was assessed by [3 H]-Thymidine incorporation after 3 days and IFN- γ release was assessed by ELISpot after 2 days in culture. The remaining cells were cloned as described above.

4.3.5 Phenotype and specificity of T-cell clones

Drug specificity was assessed by culturing autologous irradiated EBV-transformed B cells (1×10^4 /well) and flucloxacillin (1-2 mM) with T-cell clones (5×10^4 /well; 200 μ L) for 48 hours. Proliferation was measured by [3 H] thymidine incorporation (0.5 μ Ci/well, 5 Ci/ mmol, Morovek Biochemicals, Brea, CA) for the last 16 hours of culture followed by scintillation counting. Clones with a stimulation index of greater than 2 were expanded by repetitive stimulation with irradiated allogeneic PBMC (5×10^4 /well; 200 μ L) and 5 mg/mL phytohemagglutinin (PHA) in interleukin (IL)-2 containing medium (250 IU/mL). Drug-specificity of the selected T-cell clones was assessed by proliferation and ELISpot for IL13, IFN- γ , FAS ligand, perforin, and granzyme B (Mabtech). Autologous irradiated EBV transformed B cells (1×10^4 /well), flucloxacillin (1-2 mM), and T-cell clones (5×10^4 /well; 200 μ L) were incubated for 48 hours. Cell phenotyping was performed by flow cytometry on a BD FACSCanto II using CD4, CD8, CDR1, CCR2, CCR3, CCR4, CCR5, CCR8, CCR9, CCR10, CXCR3, CXCR6, and CLA antibodies (BD Biosciences) and the IO test Beta Mark TCR V β repertoire kit.

4.3.6 Migration assay

The 24-well transwell chambers were used with 5- μ m pores. A total of 0.1×10^6 T cells ($n = 4$ CD8+ clones) in 100 μ L chemotaxis buffer (RPMI 1640 + 0.5% bovine serum albumin [BSA]) were placed in the upper chambers. CCL17 (CCR4 ligand) and CCL25 (CCR9 ligand; 100 ng/ mL) in 600 μ L chemotaxis buffer were placed in the lower wells and the cells were incubated for 2 hours. Cells migrating to the lower chamber were collected and counted using a hemocytometer.

4.3.7 Mechanistic studies to characterise HLA molecules involved in the presentation of flucloxacillin to T-cells

Two assays were used to study pathways of flucloxacillin presentation to T-cell clones. First, clones (5×10^4 /well) were stimulated with autologous EBV-transformed B cells (1×10^4 /well) in the presence of anti-HLA class I and class II blocking antibodies (5 μ L; BD Biosciences, Oxford, UK); second, clones were stimulated with autologous and allogeneic EBV-transformed B cells expressing different HLA-B allotypes.

4.4 Results

4.4.1 Flucloxacillin-specific PBMC responses in patients with DILI.

Five of the six patients with DILI were positive for the risk allele HLA-B*57:01 [Table 4.2]. The remaining patient, patient number 6, expressed HLA-B*44:02 and 55:01. PBMCs were not stimulated to proliferate with flucloxacillin; however, flucloxacillin-specific PBMC responses were detected using an IFN- γ ELISpot. PBMCs from five out of the six patients, including the patient who was positive for the HLA-B alleles other than B*57:01 (patient 6), were activated with the drug [Figure 4.1-A]. The ELISpot assay was repeated with PBMC from three patients at least 1 month after the initial test and the flucloxacillin-specific response remained the same. Figures 5.1-B and C shows the dose-dependent secretion of IFN- γ and granzyme B from PBMC and a T-cell line, generated from patient 6, respectively. PBMC from flucloxacillin tolerant patients and drug-naïve volunteers were not stimulated with flucloxacillin to proliferate or secrete cytokines (data not shown).

Subject ID	HLA profile					
	HLA-A	HLA-B	HLA-C	HLA-DRB1	HLA-DQB1	HLA-DQA1
Patients with flucloxacillin-induced liver injury						
P1	02:01/29:02	07:02/57:01	06:02/07:02	15:01/07:01	03:03/06:02	01:02/02:01
P2	01:01/02:01	44:02/57:01	05:01/06:02	01:01/07:01	03:03/05:01	01:01/02:01
P3	02:01/03:01	07:02/57:01	06:02/07:02	15:01/07:01	03:03/06:02	01:02/02:01
P4	01:01/29:02	45:01/57:01	06:02/06:02	03:01/07:01	02:01/03:03	02:01/05:01
P5	01:01/29:02	08:01/57:01	06:02/07:01	03:01/07:01	02:01/03:03	02:01/05:01
P6	02:01/11:01	44:02/55:01	03:03/05:01	14:01/04:01	03:01/05:03	01:01/03:01
HLA-B*5701+volunteers used for in vitro T-cell priming						
V1	01:01/24:02	52:01/57:01	06:02/07:02	11:06/07:01	03:03/03:01	np ^a
V 2	01:01/02:01	08:01/57:01	06:02/07:01	03:01/03:01	02:01/02:01	05:01/05:01
V3	01:01/02:01	44:02/57:01	05:01/06:02	15:01/07:01	03:03/06:02	np

Table 4.2- HLA type of patients and volunteers. ^anot performed.

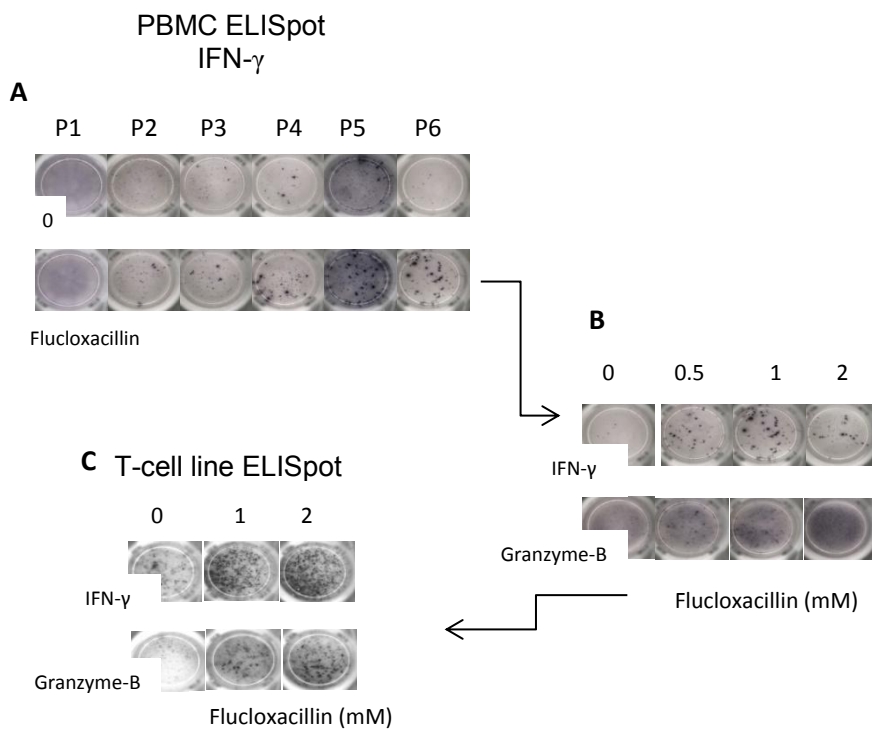


Figure 4.1- Stimulation of DILI patient lymphocytes with flucloxacillin. **(A)** Flucloxacillin-induced IFN- γ release in PBMC from patients (P1-6). **(B)** Dose response to flucloxacillin using PBMC from patient 6 in an IFN- γ and Granzyme B ELISpot. **(C)** IFN- γ and Granzyme B ELISpot for a T-cell line from patient 6.

4.4.2 Characterisation of flucloxacillin-responsive CD4+ and CD8+ T-cell clones from patients with DILI.

A total of 38 flucloxacillin-responsive T-cell clones expressing different $V\beta$ receptors were isolated from PBMC of the four IFN- γ ELISpot-positive patients expressing HLA-B*57:01. Of these, 35 were identified as CD8+ by flow cytometry [Table 4.3; 4.4]. Flucloxacillin-specific proliferation was dose-dependent, with clones displaying different response profiles up to a concentration of 2 mM. The proliferative response of CD4+ and CD8+ clones was associated with the secretion of T helper (Th) 1 and Th2 cytokines and cytolytic molecules perforin, granzyme B, and FasL [Figure 4.2]. Interestingly, the levels of cytolytic molecules secreted from the CD4+ clones was lower when CD4+ and CD8+ clones were compared.

Seven flucloxacillin-responsive CD4+ clones were isolated from patient 6, who expressed HLA-B*44:02/55:01. Over 100 other CD8+ clones were also isolated, but flucloxacillin responses were not detected.

Patient ID	HLA-B allele	PBMC ELISpot	No. of flucloxacillin-specific clones	
			CD4+	CD8+
P1	5701	-	0	0
P2-5	5701	+	3	35
P6	4402/5501	+	7	0

Table 4.3- Table summarising the phenotype of T-cell clones isolated from patients with DILI 1-6.

Clone ID	CD4+/ CD8+	vβ	Proliferation			IFN-γ ELISpot		
			0	Flucloxacillin		0	Flucloxacillin	
			cpm		Fold change	Spots		Fold change
Patients clones								
P2-50	CD8+	1	9239	48693	5.3	128	320	2.5
P2-180	CD8+	21.3	4304	54007	12.5	66	228	3.5
P2-207	CD8+	21.3	8496	20615	2.4	51	TNTC ^a	-
P2-231	CD8+	14	3793	15829	4.2	186	346	1.9
P2-58	CD8+	ND ^b	3907	34785	8.9	14	191	13.6
P5-75	CD8+	3	5357	10496	2.0	49	197	4.0
P3-2	CD4+	12	6516	75533	11.6	49	197	4.0
Volunteer clones								
V1-17	CD8+	16	2608	12075	4.6	53	300	5.7
V1-23	CD8+	8	3244	8493	2.6	26	332	12.8
V1-75	CD8+	9	3271	14156	4.3	6	149	24.8
V1-111	CD8+	21.3	2497	8546	3.4	59	309	5.2
V1-126	CD8+	14	2982	28214	9.5	41	264	6.4
V1-80	CD8+	13.2	4152	9807	2.4	11	210	19.1
V2-15	CD8+	2	4682	27004	5.8	42	204	4.9
V2-16	CD8+	1	3779	218890	57.9	28	332	11.9
V3-10	CD8+	1	4190	86916	20.7	48	TNTC	-

Table 4.4- Phenotype of the clones from HLA-B*5701+ patients with flucloxacillin-induced liver injury and HLA-B*5701 volunteers. ^atoo numerous to count; ^bnot detected.

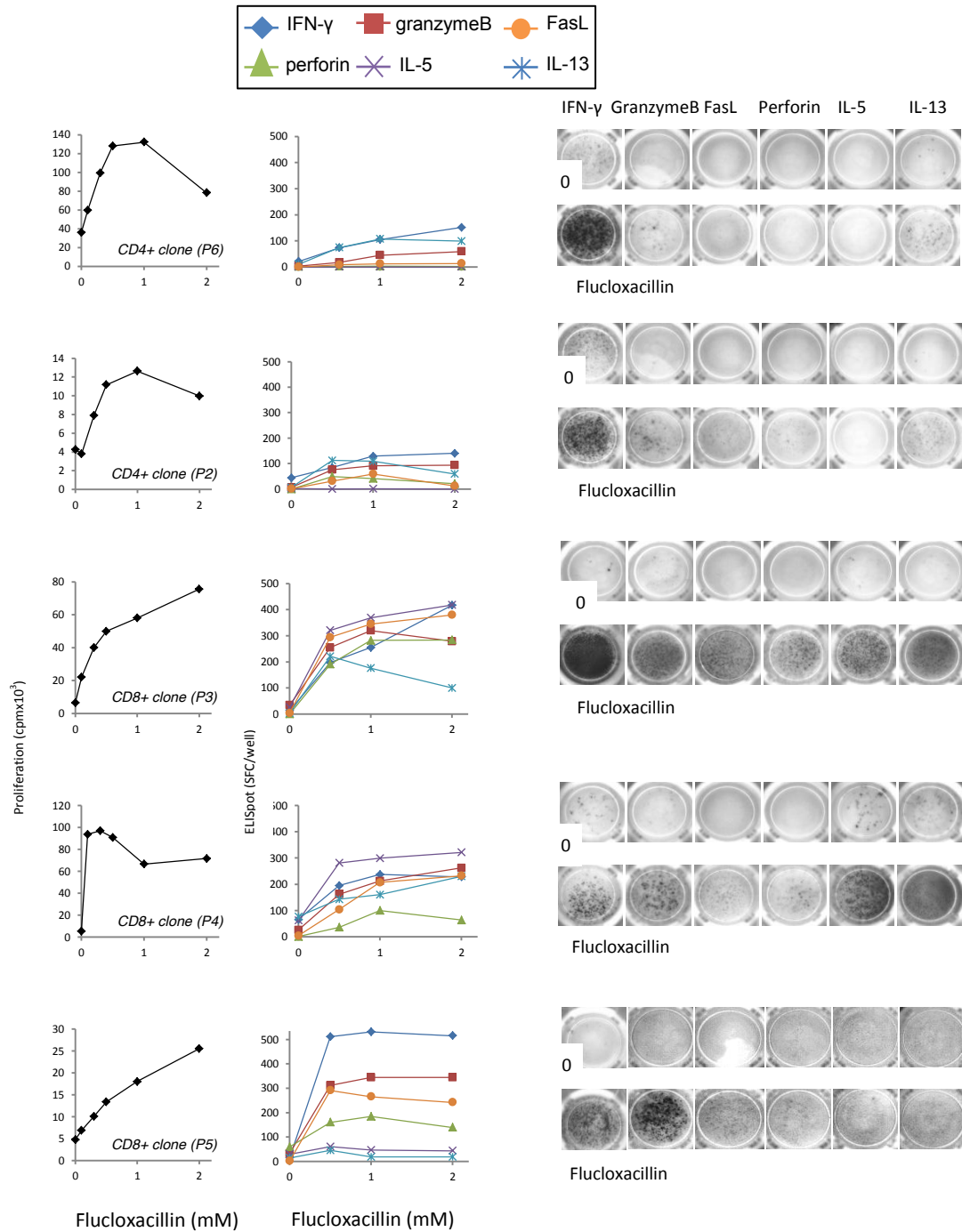


Figure 4.2- Stimulation of DILI patient T-cell clones with flucloxacillin. Proliferative response (black lines) and the secretion of cytokines and cytolytic molecules (coloured lines and pictures) from T-cell clones following flucloxacillin stimulation. Five representative clones isolated from patients 2-6 are shown. The data show the mean of replicate wells. SFC = spot forming cell.

4.4.3 Flucloxacillin activates naïve CD45RA+CD8+ T cells from volunteers expressing HLA-B*57:01.

In an attempt to prime flucloxacillin-specific T-cell responses, naïve CD3+ T cells from HLA-B*57:01-positive volunteers ($n = 3$) were cocultured with autologous dendritic cells in the presence of flucloxacillin (HLA type of the volunteers shown in Table 4.2). After 8 days the primed T cells were restimulated with fresh dendritic cells and the drug and antigen-specificity were assessed using an IFN- γ ELISpot. Low levels of IFN- γ release with flucloxacillin was detectable and the response was found to be antigen-specific and dose-dependent [Figure 4.3]. IFN- γ release was not detectable when the volunteer PBMCs were cultured with flucloxacillin. A total of 600 CD4+ and CD8+ T-cell clones were generated from the flucloxacillin-primed PBMC. Of these, 35 CD8+ clones were identified as flucloxacillin-responsive by analysis of proliferation in the presence and absence of the drug [Table 4.4]. The flucloxacillin-specific dose-dependent proliferative response and the profile of cytokines and cytolytic molecules released were similar when CD8+ clones from patients with DILI and volunteers were compared [Figure 4.4]. Flucloxacillin-responsive CD4+ clones were not detected in volunteers expressing HLA-B*57:01.

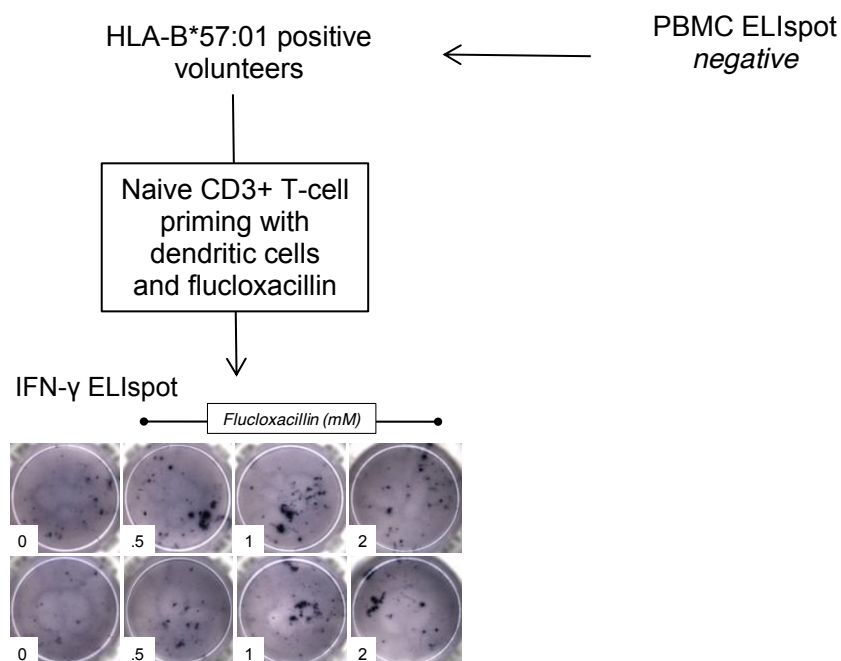


Figure 4.3- Priming of naïve T cells from HLA-B*57:01 volunteers with autologous dendritic cells and flucloxacillin. IFN- γ ELISpot after T-cell priming with flucloxacillin.

4.4.4 Flucloxacillin-responsive CD8+ T cells are restricted by HLA-B*57:01 and HLA-B*58:01.

Activation of CD8+ T-cell clones from patients with DILI and volunteers expressing HLA-B*57:01 by flucloxacillin pulsed (16h) antigen presenting cells was inhibited with an anti-HLA class I, but not a class II, blocking antibody [Figure 4.5]. The APC was pulsed with drug to permit flucloxacillin protein binding, but to limit the availability of the drug. Thus, flucloxacillin responses are dependent on the drug-derived antigen interacting with MHC class I molecules. HLA-B*57:01 restriction was studied using 16 flucloxacillin-responsive clones from patients and volunteers and antigen-presenting cells from 10 donors expressing different HLA-B molecules [Figure 4.6-A]. Six of the donors were selected based on expression of either HLA-B*57:01 or the structurally related HLA-B*58:01, which have an overlap in the peptides they display (Barber et al., 1997). Importantly, abacavir-specific T-cell responses are not detectable using antigen-presenting cells expressing HLA-B*58:01 (Chessman et al., 2008). Flucloxacillin-responsive clones were stimulated to proliferate with flucloxacillin-pulsed autologous antigen-presenting cells and antigen-presenting cells from the three donors expressing HLA-B*57:01. Furthermore, several clones were stimulated by flucloxacillin-derived antigens presented on antigen-presenting cells expressing HLA-B*58:01. Antigen-presenting cells expressing other B-alleles did not stimulate the clones [Figure 4.6-B]. Figure 4.7 shows that activation of a single clone with flucloxacillin-pulsed autologous APC, but not with APCs from 12 additional donors, each expressing different HLA-B alleles.

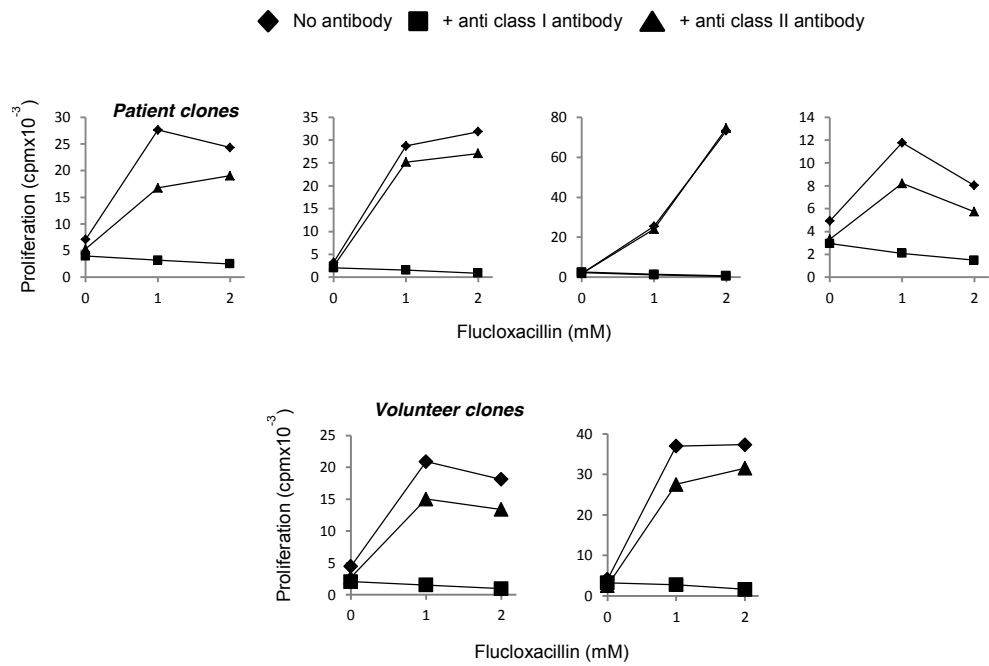


Figure 4.5- Inhibition of CD8+ flucloxacillin-specific proliferation with HLA-class I blocking antibodies in six clones using autologous antigen-presenting cells.

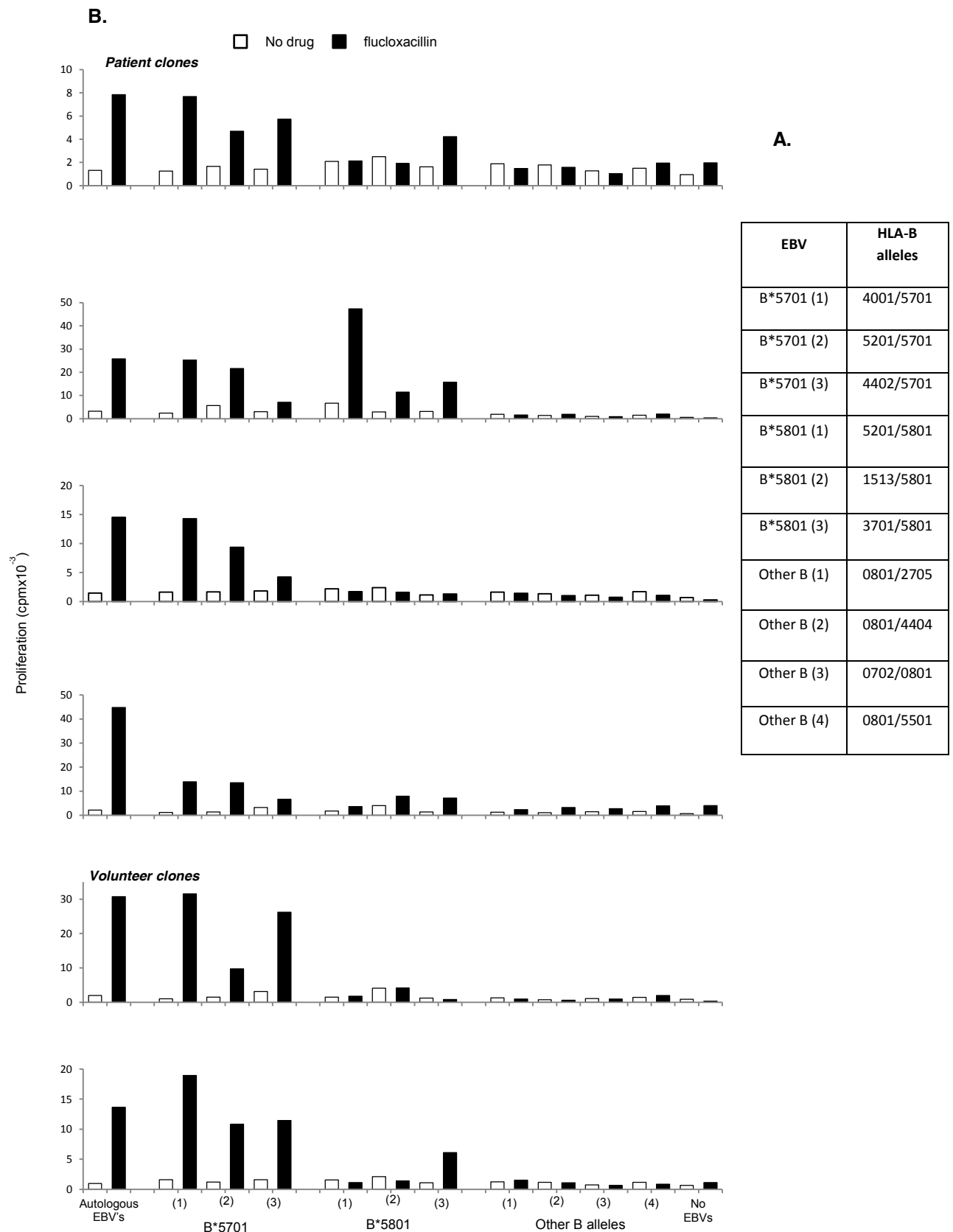


Figure 4.6- Flucloxacillin-specific stimulation of CD8+ clones is restricted by HLA-B*57:01 and B*58:01. (A) HLA-B alleles expressed by antigen-presenting cells used in (B). (B) Flucloxacillin-specific activation of six clones with flucloxacillin-pulsed (16 hours) antigen-presenting cells expressing HLA-B*57:01 and B*58:01, but not other B alleles (open bars: medium only, filled bars: 2 mM flucloxacillin). Soluble drug was not used to prevent self-presentation by MHC class I molecules expressed on the clones. Part (B) show data from different clones.

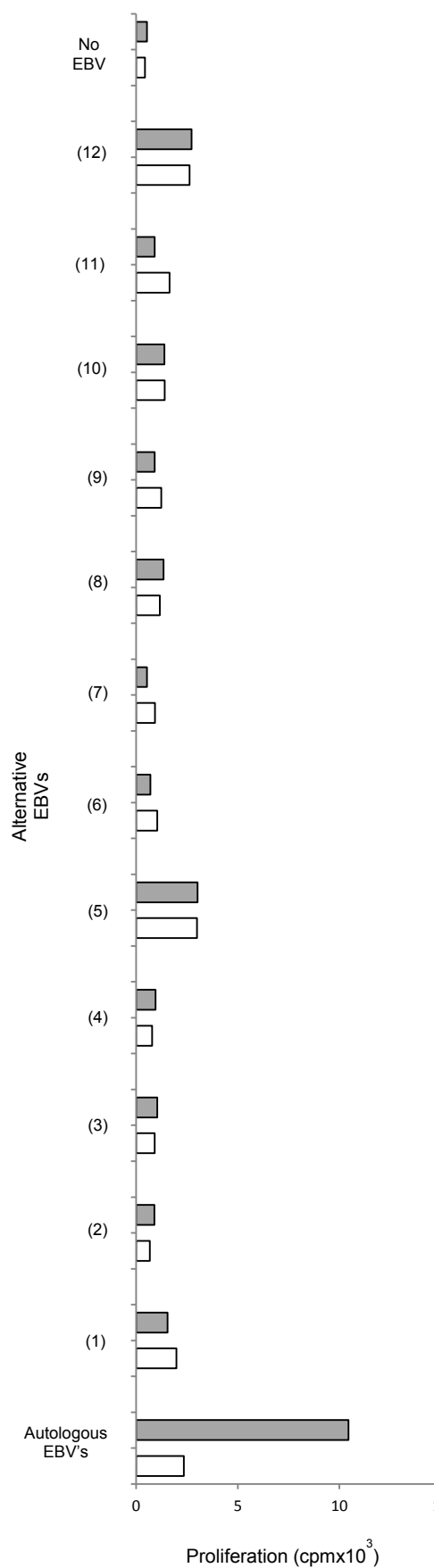


Figure 4.7- Flucloxacillin-specific activation of a CD8⁺ patient clone is restricted by HLA-B*57:01. Antigen presenting cells were pulsed with flucloxacillin for 16h (open bars: medium only, filled bars: 1 mM flucloxacillin).

EBV	HLA-B alleles
(1)	27:05/47:01
(2)	15:05/40:06
(3)	14:02/40:01
(4)	55:01/56:01
(5)	15:02/44:03
(6)	40:01/50:01
(7)	07:02/15:01
(8)	27:05/51:01
(9)	08:01/27:05
(10)	14:02/52:01
(11)	07:05/40:01
(12)	27:05/40:01

4.4.5 Expression of distinct patterns of homing receptors on flucloxacillin-specific T-cell clones.

Distinct chemokine receptor expression profiles control, at least in part, the migration of immune cells. A panel of antibodies were used to demonstrate that flucloxacillin-responsive CD8⁺ clones from patients with DILI and HLA-B*57:01-positive volunteers express high levels of the receptors CCR2, CCR4, CCR9, and CXCR3, but only low levels of CCR10 and CLA. Other chemokine receptors including CCR1, CCR3, CCR5, and CXCR6 were expressed at low levels on a limited number of clones [Table 4.5]. To show that the chemokine expression was functionally relevant, migration assays using transwells and the CCR4 and CCR9 ligands, CCL17 and CCL25, respectively, were established. Both chemokines induced the migration of CD8⁺ clones from patients with DILI and drug-naïve HLA-B* 57:01-positive subjects [Figure 4.8].

Clone ID	Patient clones						Volunteer clones					
	1	2	3	4	5	6†	1†	2	3	4	5	6†
CCR1	-*	-	1.9‡	-	-	-	-	-	1.6	-	2.0	1.6
CCR2	1.7	2.0	2.5	1.3	2.4	1.9	1.7	1.6	2.0	-	2.3	2.1
CCR3	-	-	1.6	-	-	-	-	-	-	-	-	-
CCR4	5.0	44.7	11.4	116	4.3	81.4	6.0	7.5	16.0	92.6	70.5	19.7
CCR5	1.8	-	-	-	-	-	1.8	-	1.6	-	1.8	1.9
CCR8	-	-	1.6	-	1.6	-	-	-	1.6	-	-	-
CCR9	2.9	2.9	4.1	2.1	3.4	3.2	2.3	5.9	3.9	2.5	2.0	2.9
CCR10	-	-	1.6	-	-	1.6	-	-	1.6	-	1.7	-
CXCR3	30.2	11.9	25	9.9	11.9	6.4	16.5	19.2	18.3	7.9	37.4	28.5
CXCR6	-	-	-	-	-	-	-	-	-	-	-	-
CLA	-	-	-	-	-	-	-	-	-	-	-	-

Table 4.5- Tissue homing receptors expressed on T-cell clones. *(-) indicates a value of 1.5 or less; ‡ Data presented as mean fluorescent index (fluorescence with antibody/fluorescence with isotype); † Clones also used in chemotaxis assay (see Figure 5.8).

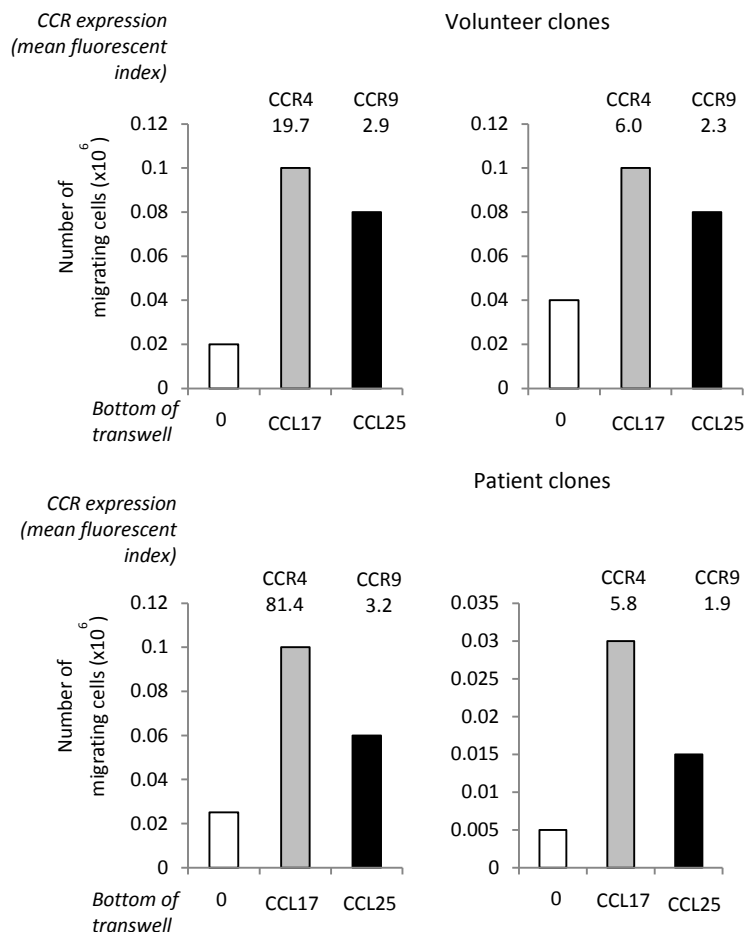


Figure 4.8- CD8⁺ clones migrate toward CCL17 and CCL25 in transwell chambers. Clones were placed in the top chamber. Chemokines were placed in the bottom chamber. After 2 hour, the number of migrated cells in the bottom chamber was counted under the microscope.

4.5 Discussion

PBMCs were isolated from six patients who developed cholestatic liver injury following flucloxacillin therapy to characterise for the first time the drug specific T-cell response. PBMC activation with flucloxacillin was detected with five patients, including a HLA-B*57:01-negative individual (P6), using ELISpot to detect antigen-driven cytokine release. Subsequently, CD4⁺ and CD8⁺ T cells were isolated from flucloxacillin-treated PBMC and cloned to characterise the cellular pathophysiology of the reaction in each patient. Flucloxacillin-responsive CD8⁺ clones expressing a range of different V β receptors were successfully isolated from the four HLA-B*57:01 ELISpot-positive patients. Contrary to the finding of Chessman et al., (Chessman et al., 2008) showing that abacavir-activated T-cells were exclusively CD8⁺, we were also successful in isolating flucloxacillin-responsive CD4⁺ clones, albeit in low numbers. Activation of clones with flucloxacillin was concentration-dependent and provoked the secretion of IFN- γ and cytolytic molecules (granzyme B, FasL, and perforin). Individuals given flucloxacillin achieve peak serum levels of 60 μ M. However, as there is significant biliary excretion, it is likely that a local concentration in the liver of 100 μ M, which activates clones, could be achieved. All of the clones expressed the chemokine receptors CCR2, CCR4, and CCR9. CCR2 and CCR9 are thought to be involved in the migration and accumulation of immune cells in the liver (Eksteen et al., 2004; Miura et al., 2012). Migration of the clones in response to CCR4 and CCR9 ligands demonstrated that the receptor expression was functionally relevant. In contrast to our previous studies with clones from patients with anticonvulsant-induced cutaneous eruptions (Homey et al., 2002; Naisbitt et al., 2003b; Wu et al., 2007), the skin homing lymphocyte receptors CCR10 and CLA were detected at low levels. Flucloxacillin-responsive clones were also isolated from the HLA-B*44:02/55:01-positive patient. However, the clones were all CD4⁺. Collectively, these data argue that immune phenomena contribute to the development of flucloxacillin-induced liver injury.

If the HLA-B*57:01 genotype is a functional determinant of flucloxacillin-induced liver injury, it should be possible to prime naïve T cells from HLA-B*57:01-positive

volunteers. To explore whether flucloxacillin activates naïve T cells, we employed our recently established T-cell priming assay that recapitulates key elements of events that occur *in vivo* during elicitation of an immunological drug reaction (Faulkner et al., 2012b). Flucloxacillin-primed T cells from HLA-B*57:01-positive volunteers were found to secrete IFN- γ following restimulation, whereas the cells that had divided were shown to be CD8⁺ by T-cell cloning. Over 30 flucloxacillin-specific CD8⁺ clones generated from three volunteers were found to proliferate and secrete cytokines and cytolytic molecules following drug stimulation. The profile of secretory molecules and chemokine receptor expression was similar to those observed with CD8⁺ clones from patients with DILI.

Genetic restriction of the flucloxacillin-specific response was studied using a panel of CD8⁺ clones from patients with DILI and HLA-B*57:01-positive drug-naïve volunteers. Activation of CD8⁺ clones was detected with flucloxacillin-pulsed antigen-presenting cells from volunteers expressing HLA-B*57:01 and B*58:01. Flucloxacillin-pulsed antigen-presenting cells expressing other HLA-B alleles did not activate the clones. Importantly, HLA-B*57:01 and B*58:01 are part of the same HLA-B17 serotype; they differ in structure by only five amino acids and have a significant overlap in their antigenic peptide repertoire (Chessman et al., 2008). Given that the HLA-B*57:01 allele is in the strongest linkage disequilibrium with DRB1*07:01 in worldwide population (Adam et al., 2012), it is not surprising that all patients positive for B*57:01 were also carriers of DRB1*07:01. We are actively pursuing whether flucloxacillin-responsive CD4⁺ T cells are DRB1*07:01-restricted.

The discovery of surprisingly strong association between the expression of HLA alleles and DILI has changed the way in which researchers view this form of iatrogenic disease. Our data characterising flucloxacillin-responsive T cells in patients represents a fundamental breakthrough in our understanding of the role of

the adaptive immune system in liver injury. Moreover, the successful priming of naïve CD8 T cells using PBMC from HLA-B*57:01-positive volunteers effectively links the genetic association to the disease pathogenesis. In ongoing studies we are seeking to investigate how and why flucloxacillin-specific T cells kill liver cells in susceptible patients.

Chapter Five

Studies to investigate mechanisms of flucloxacillin presentation to T-lymphocytes

5.1 Introduction	124
5.2 Aims	125
5.3 Patients and methods	126
5.3.1 Patients' demographics	126
5.3.2 Flucloxacillin-specific T cell clones cross reactivity	126
5.3.3 Mechanistic studies of antigen presentation	126
5.3.4 Characterisation of β -lactam albumin binding in culture	126
5.3.5 Immune epitope database analysis (IEDB) software	128
5.3.6 Statistical analysis	128
5.4 Results	128
5.4.1 Level of flucloxacillin-albumin binding at specific lysine residues	128
correlation with the processing-dependent activation of T-cell clones	
5.4.2 Additional reactivity of flucloxacillin-responsive clones against	131
alternative β -lactam antibiotics that form similar haptenic determinants on	
albumin	
5.4.3 Albumin peptide sequence incorporating the flucloxacillin binding sites	135
Lys190 and Lys212 interaction with HLA-B*57:01	
5.4.4 Proliferative response of Flucloxacillin-responsive CD8 ⁺ T cells toward	135
abacavir	
5.5 Discussion	137

5.1 Introduction

The existence of antigen-specific T cells in the peripheral blood and target organs such as skin and liver of drug hypersensitive patients provides a strong evidence for their contribution in the pathogenesis of a reaction (Brander et al., 1995; Schnyder et al., 2000; Nassif et al., 2004a; Beeler et al., 2006; Wu et al., 2007; Castrejon et al., 2010a). It is believed that T cells are activated by drugs through covalent modification of protein which lead to generation of novel antigenic determinants (Padovan et al., 1996a; Padovan et al., 1997; Beeler et al., 2006; Castrejon et al., 2010a; Elsheikh et al., 2010). However, the lack of studies that delineates the chemistry of drug-protein binding in hypersensitive patients has severely limited mechanistic studies that correlate immune function to the chemistry of antigen. Indeed, the basic theory of the hapten hypothesis of drugs hypersensitivity has been questioned by studies which reveal that drugs stimulate T cells via non-covalent interactions with MHC (Schnyder et al., 1997; Schnyder et al., 2000; Burkhart et al., 2001; Hashizume et al., 2002; Farrell et al., 2003; Naisbitt et al., 2003a; Depta et al., 2004; Nassif et al., 2004a; Keller et al., 2010).

Non-immediate hypersensitive allergic reactions to β -lactam antibiotics persist as a significant clinical issue. It is well known that β -lactam antibiotics form irreversible covalent bonds with lysine residues on protein. Then, the nucleophilic lysine residues will target the β -lactam ring. This nucleophilic attack will lead to ring opening and penicilloyl group binding (Batchelor et al., 1965b). The penicilloyl antigen may also be formed by binding of the reactive degradation product penicillenic acid (Levine, 1960). Additionally, drug-protein antigens can derive from the conversion of β -lactam antibiotics into penicilloate and penicilloic acid (Levine and Redmond, 1969). Accordingly, binding of drug-protein is thought to be an essential step to initiate an immune response and to develop the clinical signs of hypersensitivity in high risk patients (Brander et al., 1995; Padovan et al., 1996a).

Flucloxacillin is an antistaphylococcal β -lactam antibiotic that is widely prescribed in the UK and Australia. Flucloxacillin can cause a cholestatic hepatitis which might be accompanied by a rash in some cases (Olsson et al., 1992). Healthy volunteers and *in vitro* research studies have revealed that 95%-97% of flucloxacillin is bound to plasma protein which is mainly albumin (Roder et al., 1995). Therefore, substituting flucloxacillin with alternate antistaphylococcal agents such as dicloxacillin, oxacillin, and cloxacillin should be considered. However, the structural similarities between these antistaphylococcal agents might lead to cross-reactivity (Baldo, 1999) which required further studies.

Mass spectrometry methods have been recently developed in Liverpool to characterise the amino acids residues modified with drugs (Callan et al., 2009a; Jenkins et al., 2009a). Therefore, it was possible to further investigate the functional antigens formed from flucloxacillin in *ex vivo* cultures with T cells from DILI patients in order to correlate protein modification to drug antigenicity and immunogenicity. I acknowledge Dr Roz Jenkins from the proteomic team (Principal Experimental Officer) as all samples were analysed on the mass spectrometer by her.

5.2 Aims

The aim of this work was to;

- Characterise the mechanisms of flucloxacillin antigen presentation to T cells.
- Investigate T-cell receptor cross reactivity of the flucloxacillin-responsive T-cell clones with the different β -lactam antibiotics.

5.3 Patients and methods

5.3.1 Patients' demographics

Six patients with flucloxacillin-induced liver injury and six flucloxacillin-exposed tolerant controls were enrolled for this study. Detailed clinical history on patients with flucloxacillin-induced liver injury is presented in chapter 4 [Table 4.1].

5.3.2 Flucloxacillin-specific T cell clones cross reactivity

T-cell clones were tested for reactivity against piperacillin, penicillin G, amoxicillin, oxacillin, cloxacillin, and dicloxacillin (all 0.1-2) mM, nitroso sulfamethoxazole (10-100 μ M), and abacavir (10-100 μ M). Detailed method is described in chapter 2. Dose ranges have been shown to be optimal for the activation of T cells.

5.3.3 Mechanistic studies of antigen presentation

Autologous EBV-transformed B cells were subjected to glutaraldehyde fixation (0.05%) (Sigma-Aldrich, Gillingham, Dorset, UK) to terminate metabolic process and/or were incubated for 1, 4, 16, or 48 hours with flucloxacillin (2 mM) followed by three washes to remove soluble drug.

5.3.4 Characterisation of β -lactam albumin binding in culture

To identify the key drug-modified lysine residues in albumin, we utilized our recently described mass spectrometry methods (Jenkins et al., 2009a; Whitaker et al., 2011a; El-Ghaiesh et al., 2012). Flucloxacillin was incubated with EBV transformed B cells in RPMI 1640 medium containing 10% human AB serum for 1-48 hours. At each timepoint the cells were removed by centrifugation at 450g. Serum proteins were precipitated from culture supernatant by the addition of nine volumes of ice-cold methanol followed by centrifugation at 14,000g and 4° C for 15 minutes. Flucloxacillin, piperacillin, penicillin G, amoxicillin oxacillin, cloxacillin, and

dicloxacillin were also incubated with human serum albumin at a molar ratio of drug to protein of 10:1 for 16 hours and methanol precipitated.

Prior to mass spectrometry, all samples were reduced and alkylated before again being subjected to methanol precipitation. They were reconstituted in ammonium bicarbonate buffer (50 mM), digested with trypsin overnight at 37° C, and then desalted using C₁₈ Zip-Tips (Millipore). Samples (2.4-5 pmole) were delivered into a QTRAP 5500 hybrid quadrupole-linear ion trap mass spectrometer (ABSciex) by automated in-line liquid chromatography (U3000 HPLC System, 5 mm C₁₈ nanoprecolumn, and 75 μ m x 15 cm C₁₈ Pep-Map column [Dionex, Sunnyvale, CA]) by way of a 10- μ m inner diameter PicoTip (New Objective, Woburn, MA). A gradient from 2% ACN/0.1% FA (v/v) to 50% ACN/0.1% FA (v/v) in 70 minutes was applied at a flow rate of 280 nL/min. The ionspray potential was set to 2,200–3,500V, the nebulizer gas to 18, and the interface heater to 150° C. Established multiple reaction monitoring (MRM) transitions specific for drug-modified peptides were employed. MRM survey scans were used to trigger enhanced product ion dual mass spectrometry (MS/MS) scans of drug modified peptides. Total ion counts were determined from a second aliquot of each sample analysed by conventional liquid chromatography (LC)-MS/MS on the same instrument and were used to normalise sample loading on column. MRM peak areas were determined by MultiQuant 1.2 software (ABSciex). The relative intensity of MRM peaks for each of the modified lysine residues within a sample were compared and were normalized across samples. All samples were analysed on the mass spectrometer by Dr Roz Jenkins (Principal Experimental Officer).

5.3.5 Immune epitope database analysis (IEDB) software

The MHC class I T cell epitope prediction tool on the IEDB website (http://tools.immuneepitope.org/main/html/tcell_tools.html) was used to predict which peptides from human serum albumin were likely to be high affinity binders to HLA-B*57:01. The IEDB recommended prediction method was used, the relevant allele was selected, and all lengths of peptides were allowed. Peptides with median inhibitory concentrations (IC₅₀s) of <50 nM (high affinity binders) or <500 nM (intermediate affinity binders) containing lysine residues modified by all of the β -lactams studied were listed.

5.3.6 Statistical analysis

Student *t* test was used to analyse the proliferation and ELISpot data.

5.4 Results

5.4.1 Level of flucloxacillin-albumin binding at specific lysine residues correlates with the processing-dependent activation of T-cell clones

To investigate the role of drug protein binding in the generation of flucloxacillin-derived antigens for T cells, antigen-presenting cells were pulsed with the drug for 1,4,16, and 48 hour, prior to washing and exposure to clones. A 16 to 48-hour incubation period was required to stimulate a reproducible proliferative response with all clones [Figure 5.1-A] and the strength of the response was similar to that seen with the soluble drug.

Mass spectrometric analysis of albumin in flucloxacillin-treated cell cultures revealed an irreversibly bound hapten of the predicted mass of 453 amu, which was formed from direct adduction of flucloxacillin. After 48 hours, 12/59 lysine residues were modified, including Lys190 and Lys212, which are modified on albumin isolated from plasma of all flucloxacillin-exposed patients (Jenkins et al., 2009a). Adduct formation on albumin at each modified Lys residues was dependent on

incubation time [Figure 5.1-B] and a strong positive correlation between the level of flucloxacillin binding and the strength of the proliferative response stimulated by flucloxacillin-pulsed antigen-presenting cells was observed [Figure 5.1-C]. Glutraldehyde fixation of antigen-presenting cells, which inactivates protease enzyme activity and antigen processing, inhibited the proliferation of clones against flucloxacillin-pulsed antigen-presenting cells [Figure 5.1-D].

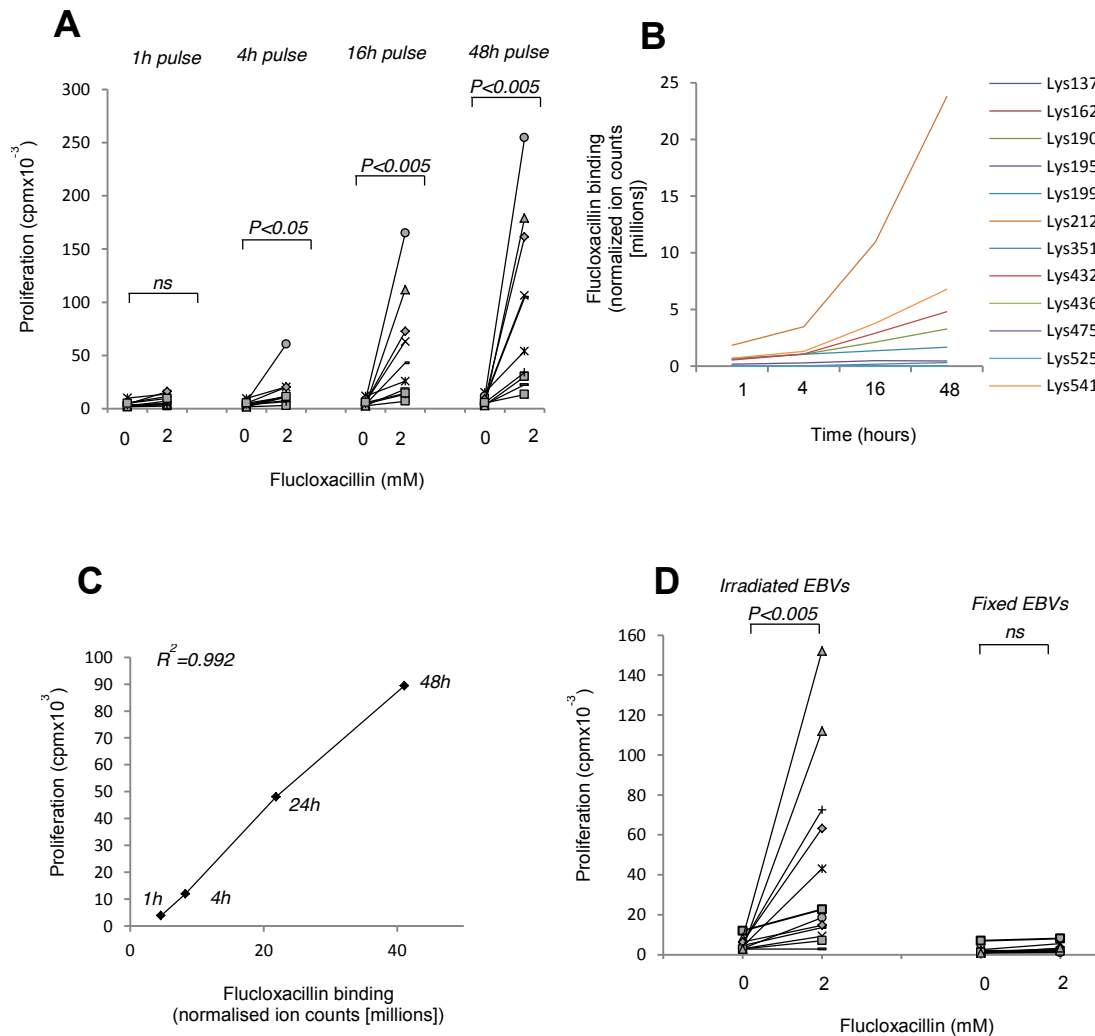
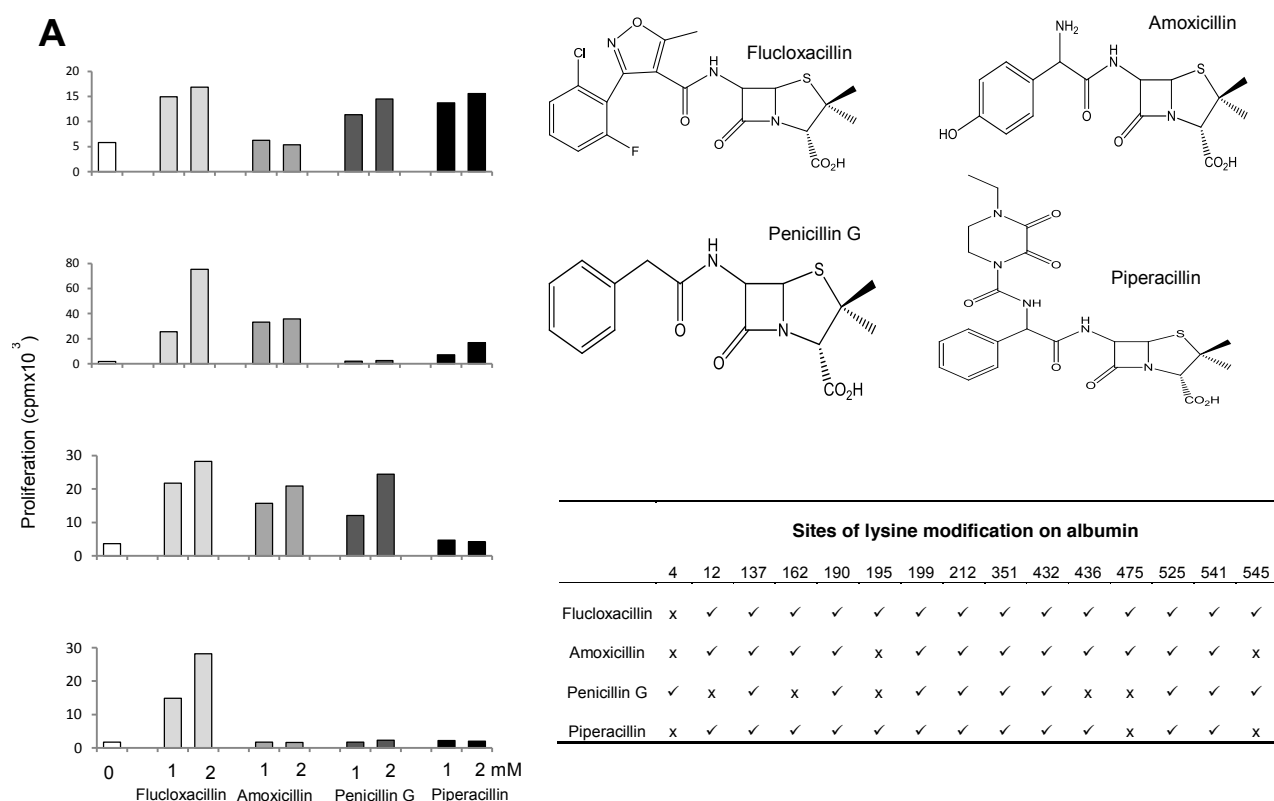


Figure 5.1- Flucloxacillin binds irreversibly to protein to stimulate T-cell clones. **(A)** Stimulation of a panel of 10 clones with flucloxacillin-pulsed antigen-presenting cells. Antigen-presenting cells were washed repeatedly after pulsing to remove unbound drug. The data shows the mean of replicate wells. Results were analysed by Student's *t* test. **(B)** Flucloxacillin binds to multiple lysine residues on serum albumin in a time-dependent manner in cell culture, as detected by mass spectrometry. **(C)** Correlation between the time-dependent relative level of flucloxacillin covalent binding to albumin in serum-supplemented culture medium and the strength of the mean drug-specific T-cell response ($n = 10$ clones; cpm in control wells subtracted). **(D)** Stimulation of a panel of 10 clones with flucloxacillin and irradiated or glutaraldehyde-fixed antigen-presenting cells. Fixation blocks protein processing. The data show the mean of replicate wells. Results were analysed by Student *t* test.

5.4.2 Flucloxacillin-responsive clones display additional reactivity against alternative β -lactam antibiotics that form similar haptenic determinants on albumin.

To study chemical restriction of the flucloxacillin-specific T-cell response, clones were cultured with antigen-presenting cells and 4 β -lactam antibiotics (flucloxacillin, piperacillin, amoxicillin, and penicillin G) and hapten albumin binding profiles and proliferation were measured. Greater than 80% of the clones displayed additional reactivity against at least one β -lactam, which form similar haptenic determinants with Lys residues on albumin. In fact, drug modifications were detectable at eight Lys residues (Lys132, Lys190, Lys199, Lys212, Lys351, Lys432, Lys525, and Lys541) with all four drugs. Figure 5.2 shows the drug structures, the proliferative response of four representative clones that show the different crossreactivity profiles observed, and the sites of lysine modification on albumin. Figure 5.3 shows the dose-dependent proliferative response of clones with optimal stimulatory concentrations of the different drugs.

In subsequent experiments, hapten albumin binding profiles and T-cell responses were studied with oxacillin, cloxacillin, and dicloxacillin [Figure 5.4-A], which are used in certain countries as an alternative to flucloxacillin. Hapten modifications were detected on the same 12 lysine residues with all four drugs and the level of modification at each site was comparable [Figure 5.4-B]. Furthermore, all of the flucloxacillin-responsive clones tested displayed reactivity against the structurally related drugs [Figure 5.4-C].



B

Prediction method: IEDB recommended | Low percentile = good binders

Allele	Start	End	Peptide Length	Sequence	Method used	Percentile Rank	IC50 (nM)	Lysine
HLA-B*57:01	212	219	8	KAWAVARL	NetMHCpan	0.5	396.76	212
HLA-B*57:01	209	219	11	RAFKAWAVARL	NetMHCpan	0.5	427.98	212
HLA-B*57:01	190	199	10	KASSAQRLK	SMM	0.6	72.68	190, 199
HLA-B*57:01	191	200	10	ASSAQRLKC	SMM	2	228.03	199
HLA-B*57:01	538	547	10	KATKEQLKAV	SMM	2	216.05	432, 436

Lys190 Lys199 Lys212

...QAADKAACLLPKLDDELRLDEG**KASSAQRLK**CASLQKFGER**AFKAWAVAR**LSQRFPKAEFAEVSK....

Bold text – tryptic peptide
Large text – MHC binding peptide
Red text – flucloxacillin-modified Lys

Figure 5.2- Flucloxacillin binds irreversibly to protein to stimulate T-cell clones. **(A)** Variable crossreactivity of flucloxacillin-specific clones with related β -lactam antibiotics and mass spectrometric analysis of the profile of lysine modification with each drug *in vitro* (table shows Lys residues modified after 16 hours [10:1; drug: albumin]). Four representative clones that illustrate the different response profiles are shown (see Figure 5.3 for dose-response curves). The data show the mean of replicate wells. **(B)** Identification of peptides from serum albumin with high binding affinity to HLA-B*57:01, which contain lysine residues consistently modified with the crossreactive β -lactam antibiotics. IEDB analysis software was used to identify the peptides.

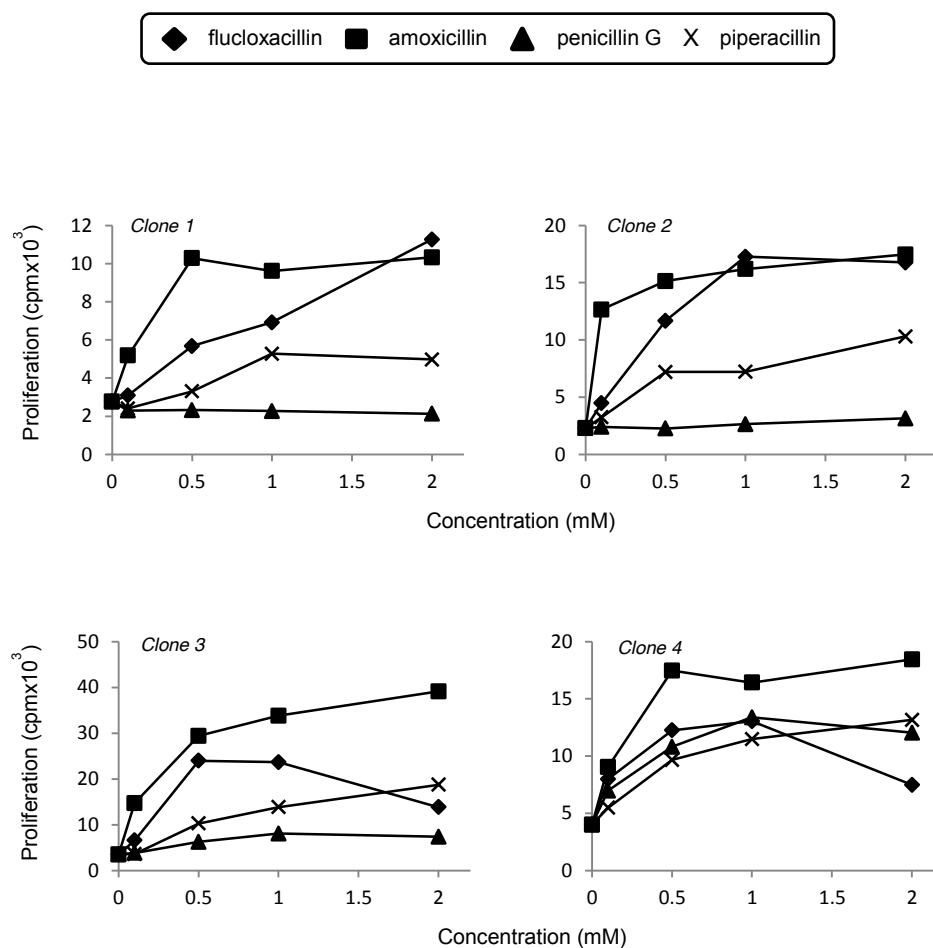


Figure 5.3- Concentration-dependent activation of CD8+ T-cell clones with flucloxacillin, amoxicillin, penicillin G, and piperacillin. Proliferation was measured by incorporation of [³H]-thymidine. Results show mean cpm in the presence and absence of drug.

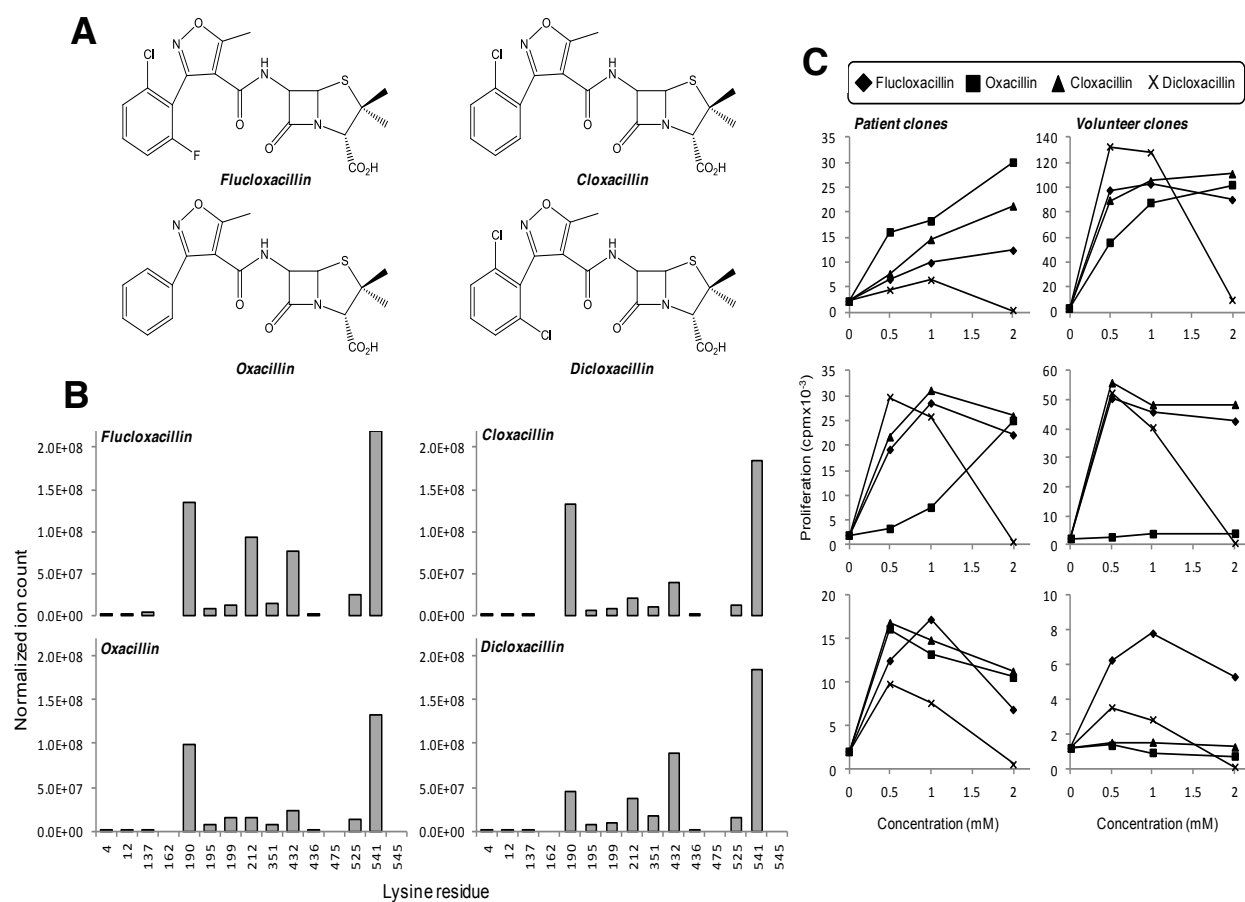


Figure 5.4- Flucloxacillin-responsive CD8⁺ clones are stimulated with oxacillin, cloxacillin, and dicloxacillin, which, bind irreversibly to similar lysine residues on albumin. **(A)** Structures of the drugs. **(B)** Mass spectrometric analysis of the profile of lysine modification on albumin with each drug *in vitro* after 16 hours (10:1; drug: albumin). **(C)** Stimulation of six flucloxacillin-responsive T-cell clones with oxacillin, cloxacillin, and dicloxacillin. Proliferation was measured by incorporation of [³H]-thymidine. Results show mean cpm in the presence and absence of drug.

The drug metabolite nitroso sulfamethoxazole, which binds irreversibly to cysteine residues on multiple proteins generating antigenic determinants for Tcells, was used as a specificity control. Flucloxacillin-responsive clones were not activated with the nitroso metabolite [Figure 5.5-A].

5.4.3 Albumin peptide sequence incorporating the flucloxacillin binding sites Lys190 and Lys212 interact with HLA-B*57:01

The Immune Epitope Database Analysis Resource was utilised to explore potential high affinity HLA-B*57:01 binding peptides derived from human serum albumin. Several peptide sequences that contain flucloxacillin-modifiable Lys residues were identified as high affinity HLA-B*57:01 binders [Figure 5.2-B], including Lys190 and Lys212, which are targets for flucloxacillin-derived haptens in drug-exposed patients.

5.4.4 Flucloxacillin-responsive CD8+ T cells are not activated with abacavir

The drug-specific T-cells response in abacavir hypersensitive patients is exclusively HLA-B*57:01 restricted; thus, we conducted experiments to determine whether flucloxacillin-responsive clones are activated with abacavir. All 14 clones tested proliferated in the presence of flucloxacillin, but not abacavir [Figure 5.5-B].

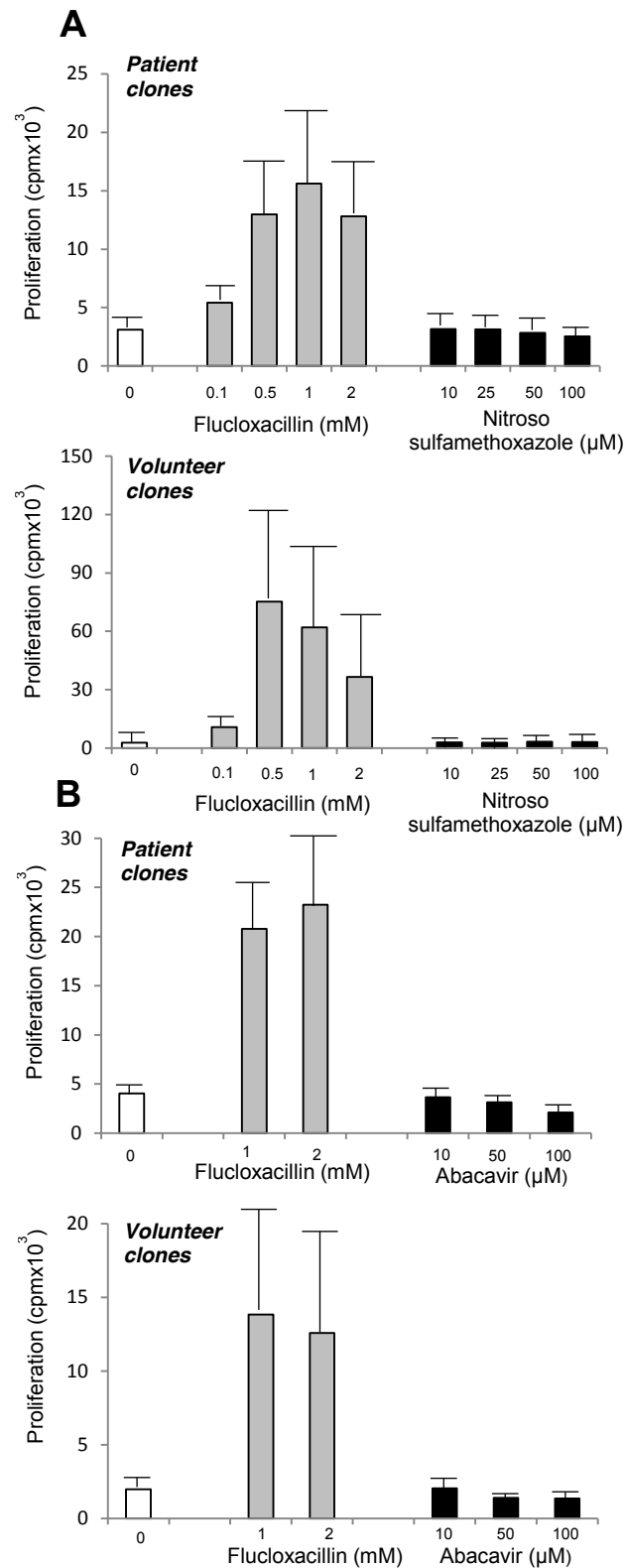


Figure 5.5- Flucloxacillin-responsive HLA-B*57:01 restricted CD8+ clones from patients and volunteers are not stimulated with nitroso sulfamethoxazole or abacavir. **(A)** Stimulation of 7 flucloxacillin-responsive T-cell clones (4 from patients with liver injury; 3 from volunteers) with flucloxacillin and nitroso sulfamethoxazole. **(B)** Stimulation of 14 flucloxacillin-responsive T-cell clones (7 from patients with liver injury; 7 from volunteers) with flucloxacillin and abacavir. Proliferation was measured by incorporation of [³H]-thymidine. Results show mean cpm±SD in the presence and absence of drug.

5.5 Discussion

It is well established that an obligatory step in β -lactam allergy is the formation of covalent bonds between drug and lysine residues on protein. The β -lactam ring is targeted directly by nucleophilic lysine residues. Nucleophilic attack leads to ring opening and binding of the penicilloyl group. The protein conjugate can also be formed through binding of the reactive degradation product penicillenic acid. Using mass spectrometric methods, we recently identified albumin as a major circulating protein modified with β -lactam antibiotics including flucloxacillin, defined the profile of drug protein conjugation at specific lysine residues with respect to dose and incubation time, and characterised for the first time the sites of modification associated with the stimulation of a clinically relevant drug-specific T-cell response (Jenkins et al., 2009a; Whitaker et al., 2011a; El-Ghaiesh et al., 2012). Herein, we show that (1) the stimulation of clones with flucloxacillin-pulsed antigen-presenting cells, and (2) the detection of flucloxacillin haptens on albumin in culture are time-dependent. Furthermore, simultaneous measurement of antigenicity and immune responsiveness revealed that the cumulative level of flucloxacillin protein binding at each timepoint studied correlated directly with the strength of the T-cell proliferative response. Aldehyde fixation of antigen-presenting cells, which inhibits processing, blocked the flucloxacillin-specific stimulation of clones [Figure 5.1-D]. Collectively, these data indicate that flucloxacillin-protein binding is critical for the formation of functional T-cell antigens. Interestingly, the β -lactam antibiotics piperacillin, amoxicillin, and penicillin G, which bind to similar albumin lysine residues, also stimulated the flucloxacillin-responsive clones. The detection of broadly crossreactive T cells is in contrast to our recent studies describing highly drug-specific T-cells in piperacillin-hypersensitive patients (see chapter 2), and suggests that MHC binding peptides and the core penicilloyl structure provide the binding energy to drive T-cell responses in patients with flucloxacillin-induced liver injury.

Carey and van Pelt (Carey and van Pelt, 2005) raised an antibody specific for flucloxacillin-modified proteins to characterise whether flucloxacillin treatment results in adduct formation *in vivo*. Interestingly, they found that western blot analysis of liver cytosol from treated rats revealed a single-flucloxacillin-modified

band with a weight of ~66 kDa, (i.e., the molecular weight of albumin). Thus, in our next series of experiments the IEDB analysis resource was used to identify HLA-B*57:01 binding peptides derived from albumin. Peptide sequences containing Lys190, Lys199, and Lys212 were identified as high affinity binders at HLA-B*57:01. Interestingly, all the penicillins investigated modify albumin at these residues.

Previous studies have reported that it may be feasible to substitute flucloxacillin with an alternative anti-staphylococcal agent such as oxacillin, cloxacillin, or dicloxacillin in HLA-B*57:01-positive individuals (Daly et al., 2009). Our data shows remarkably consistent albumin binding profiles and stimulation of HLA-B*57:01-restricted CD8⁺ clones across the different drugs. Thus, loss of fluorine, which has the same volume as hydrogen, and chlorine, does not alter the binding of flucloxacillin to protein or indeed the assembly of haptenic determinants that stimulate T cells. This clearly highlights a potential risk of using alternative oxacillins in HLA-B*57:01-positive patients with liver injury.

Abacavir has recently been shown to interact with amino acid residues located deep within the HLA-B*57:01 (but not HLA-B*58:01) binding groove and alter the repertoire of self-peptides that are presented to CD8⁺ T cells (Chessman et al., 2008; Alfievic et al., 2012; Illing et al., 2012; Norcross et al., 2012; Ostrov et al., 2012). In contrast, flucloxacillin does not alter peptide binding to HLA-B*57:01 (Norcross et al., 2012), which supports our hypothesis that functional flucloxacillin antigens derive from naturally processed drug-protein conjugates. Flucloxacillin-responsive CD8⁺ clones from patients with liver injury and HLA-B*57:01-positive volunteers were not stimulated with abacavir. Furthermore, abacavir-responsive CD8⁺ clones generated from the same volunteers were not activated with flucloxacillin (results not shown).

Collectively, these data demonstrate that the different chemistries associated with these two drugs result in the presentation of unique HLA-B*57:01-restricted epitopes to T cells.

Chapter Six

Final Discussion

β -lactam antibiotics are considered the cornerstone of therapy for the treatment of many bacterial infections. They work by inhibiting bacterial cell wall synthesis. Critical to their mechanism of action is chemical reactivity. The β -lactam nucleus binds irreversibly to amino acid residues within the active site of penicillin binding proteins – a group of bacteria cell wall proteins with high affinity for penicillin - preventing crosslinking of the nascent peptidoglycan layer, disrupting cell wall synthesis. Unfortunately, the intrinsic reactivity of β -lactam antibiotics leads to covalent modification of other endogenous proteins, in particular lysine residues and such drug-protein adducts are thought to be involved in a variety of adverse events including hypersensitivity. Nucleophilic attack of the amino acid leads to ring opening and binding of the penicilloyl group. The penicilloyl moiety can also be formed through binding of the reactive degradation product penicillenic acid. Furthermore, drug-protein adducts derive from spontaneous conversion of β -lactam antibiotics into penicilloic acid and penicilloate (Levine, 1960; Levine and Ovary, 1961; Batchelor et al., 1965a; Pullen et al., 1968; Chang et al., 2012). Surprisingly, under cell culture conditions, adduct formation by the β -lactam antibiotics is incredibly protein-specific. All of the drugs analysed to date (penicillin G, amoxicillin, flucloxacillin, piperacillin) modify extracellular protein, but essentially ignore cellular protein (Meng et al., 2011; Whitaker et al., 2011a; El-Ghaiesh et al., 2012; Monshi et al., 2013). This protein selectivity is restricted to the β -lactam antibiotics, other reactive chemicals and drug metabolites modify multiple cellular and extracellular proteins (Naisbitt et al., 2002; Pickard et al., 2007; Sanderson et al., 2007; Callan et al., 2009b; Megherbi et al., 2009; Pickard et al., 2009; Castrejon et al., 2010b). Using novel mass spectrometric methods, we identified albumin as the major protein modified with the drugs and defined the profile of drug protein conjugation at specific lysine residues with respect to dose and incubation time. Since human serum albumin accounts for the majority of serum-bound penicilloyl groups *in vivo* (Pickard et al., 2009), we also considered it a suitable model for investigating the chemistry underlying β -lactam adduct formation in patients. The β -lactam antibiotics piperacillin and flucloxacillin were found to bind to the same

lysine residues that are modified *in vitro* (Jenkins et al., 2009a; Whitaker et al., 2011a). These data confirm that drug protein adducts are formed in exposed patients and hence have the potential to be recognized by the host immune system. However, since adducts were detected in 100% of exposed patients, factors in addition to antigen formation are needed to translate the antigenic signal into a pathogenic immune response.

The presence of drug-specific T-cells in blood and target organs of hypersensitive patients provides a robust case for their involvement in the pathogenesis of the reaction. Previous studies have characterized CD4⁺ and CD8⁺ clones responsive to a range of drug and chemical allergens including *p*-phenylenediamine, anti-convulsants, anti-bacterial agents and anti-HIV drugs (Schnyder et al., 2000; Naisbitt et al., 2003a; Naisbitt et al., 2003b; Naisbitt et al., 2005; Wu et al., 2006; Wu et al., 2007; Elsheikh et al., 2010; Jenkinson et al., 2010; Whitaker et al., 2011a). β -lactam antibiotics stimulate CD4⁺ and CD8⁺ T-cells from hypersensitive patients to proliferate and secrete a diverse cytokine secretion profile (Hertl et al., 1993c; Mauri-Hellweg et al., 1996; Brugnolo et al., 1999; Sachs et al., 2002; Beeler et al., 2006; Rozieres et al., 2009). Brander et al. (Brander et al., 1995) demonstrated that penicillin conjugated directly to MHC and synthetic penicillin-albumin constructs stimulate T-cells from allergic patients. Penicillin peptide conjugates designed to fit in specific MHC molecules have also been shown to stimulate specific T cell clones (Padovan et al., 1996b); however, experiments that relate the number of modified amino acid residues on albumin to the stimulation of specific T-cells have not been performed.

The aim of this thesis was to explore the cellular and chemical basis of two very different forms of β -lactam hypersensitivity reaction, piperacillin-induced skin injury in patients with cystic fibrosis and flucloxacillin-induced liver injury. The availability of samples collected from well-defined clinical cohorts and a cell bank of

HLA-typed drug naïve volunteers enabled us to delineate similarities and differences between the two pathogenic drug antigen-specific immune responses.

Hypersensitivity reactions to β -lactam antibiotics such as piperacillin were first reported in patients with cystic fibrosis in 1970. The majority of β -lactam reactions in patients with cystic fibrosis are non-immediate; mean time of onset 9.1 days. An accelerated response is often seen following re-exposure. Reactions consist of maculopapular rashes, fever and/or flu-like symptoms. This clinical pattern suggests an immunological event rather than direct toxicity; however, unlike β -lactam reactions in patients without cystic fibrosis, an immune pathogenesis has not been confirmed by skin testing or *in vitro* analysis of drug-specific T-cell responses. The prevalence of hypersensitivity reactions in patients with cystic fibrosis, the appearance of multi-drug resistant organisms and the cost of new drugs makes it increasingly difficult to treat infective episodes effectively. Patients often have to be admitted to hospital disrupting education, work and family life. When a patient has had a history of a previous hypersensitivity reaction they need desensitizing to the drug through a dose escalation regimen at the start of every treatment course. Thus, it is especially important to understand mechanisms relating to the development of hypersensitivity in patients with cystic fibrosis.

In initial experiments the lymphocyte transformation test was used to identify drug-specific lymphocyte proliferative response in approximately 70% of clinically-diagnosed piperacillin hypersensitive patients. The response was drug concentration dependent and associated with the secretion of cytokines and cytolytic molecules. Importantly, piperacillin-specific responses were not detected in drug-tolerant and drug-naïve controls with cystic fibrosis. These data suggest that it might be possible to administer piperacillin at therapeutic doses to hypersensitive patients with a negative lymphocyte transformation test; however, for this assay to be used diagnostically in the cystic fibrosis unit an improved understanding of drug-induced hypersensitivity reactions is required. Thus, Liverpool, in collaboration with the cystic fibrosis unit in Leeds, has recently initiated the first longitudinal study of drug hypersensitivity to probe the multiple pathways that regulate cellular immune

responses and delineate the key “switches” that determine whether drug exposure will be associated with the development of a hypersensitive or tolerant response. Patient samples are being collected prior to a reaction, in the acute phase and longitudinally as the patient recovers. Since these studies with patient PBMC provide limited information on the mechanism of drug antigen presentation and the phenotype and function of piperacillin-specific T-cells, T-cells were cloned from a cohort of hypersensitive patients with positive lymphocyte transformation test results. The majority of piperacillin-responsive T-cells were CD4⁺ and stimulation of the clones was highly drug-specific. These data indicate that the piperacillin side-chain confers specificity at the MHC T-cell receptor interface as each of the drug structures tested contained the same core β -lactam nucleus. The activation of clones was drug concentration-dependent. Furthermore, T-cell responses were only detected after a culture period of 4-16h. These data indicate that the activation of clones is dependent on the degradation of the drug and irreversible modification of protein lysine residues or lysine residues found on MHC-binding peptides displayed on the surface of antigen presenting cells. To determine whether clones are activated via a hapten mechanism, a piperacillin-albumin adduct was synthesized with piperacillin bound to the same lysine residues modified in patient plasma and in cell culture. This conjugate was found to activate clones and the T-cell response was dependent on protein processing.

Interestingly, the time-dependent activation of piperacillin clones explained why it was difficult to detect T-cell-mediated killing of ⁵¹Cr-loaded autologous target cells. The standard ⁵¹Cr-release assay is analysed 4h after drug antigen exposure. By pre-treating antigen presenting cells with piperacillin for 16h, drug-specific T-cell mediated killing was readily detectable.

Multiple hypersensitivity reactions in patients with cystic fibrosis is an important clinical problem. Thirty per cent of hypersensitive patients developed reactions to

more than 2 drugs. To investigate whether this clinical phenomenon relates to the activation of naïve T-cells against different β -lactam antibiotics or T-cell receptor crossreactivity, PBMC responses were analysed in patients hypersensitive to the drugs piperacillin, meropenem and aztreonam and clones were generated to study crossreactivity. PBMC proliferative responses and cytokine release were detectable when PBMC were cultured with the different drugs. T-cell clones responsive against each drug were generated successfully from several hypersensitive patients; however, crossreactivity with the different compounds was not observed. Importantly, each drug formed a structurally distinct hapten, but bound to similar lysine residues on human serum albumin – albeit at different levels; hence, the different haptenic structures provide the chemical basis for the highly drug specific T-cell response detected in patients with multiple hypersensitivity. Interestingly, Daubner et al (Daubner et al., 2012) found that in patients with multiple drug hypersensitivity the drug-responsive T-cells are contained in a pre-activated T-cell fraction (CD38^{high} [marker for cell activation]; PD-1^{high} marker for cell exhaustion)]; thus, as part of our longitudinal study, we are measuring these markers to explore whether this phenotype is a common characteristic of the drug-specific T-cell response in this patient group.

In stark contrast to piperacillin, human exposure to flucloxacillin is associated with liver injury. The incidence of flucloxacillin-induced liver injury has been estimated at 8.5 in 100000 new users (Daly and Day, 2012). The majority of reactions are non-immediate; symptoms are detected 1-45 days after starting treatment. Recently, Daly et al. (Daly et al., 2009) detected a strong association between susceptibility to flucloxacillin DILI and expression of HLA-B*5701, which implies a direct effect of the gene product on the disease pathogenesis. These findings suggest that a major susceptibility factor relates to the restriction of the fit of the antigen into particular immunological receptors in an appropriate chemical form. Flucloxacillin is known to form an antigen in patients and *in vitro* through the irreversible modification of specific lysine residues on albumin (Jenkins et al., 2009b); however, drug-responsive T-cell responses in patients with flucloxacillin-induced liver injury have not been

described. In fact, little is known about the role of the adaptive immune system in reactions targeting the liver. In 1997, Maria and Victorino (Maria and Victorino, 1997) described lymphocyte responses to drugs in over 50% of patients with drug-induced liver injury. However, the phenotype and function of T-cells and the mechanisms of antigen presentation have not been described. Thus, the final aim of my thesis was to characterize the cellular response in patients with flucloxacillin-induced liver injury, investigate whether it is possible to prime naïve T-cell against flucloxacillin using PBMC from volunteers carrying the risk allele HLA-B*57:01 and study mechanisms of flucloxacillin-specific T-cell activation.

In initial experiment, somewhat disappointingly, the lymphocyte transformation test yielded negative results. Flucloxacillin-responsive T-cells were not detectable in patients with liver injury or drug-exposed controls. However, when the end-point of the PBMC assay was changed to an ELISpot, the flucloxacillin-specific secretion of cytokines and cytolytic molecules was detected in 5 out of 6 DILI patients. These data support the work of Rozieres et al., (Rozieres et al., 2009) who found that IFN- γ ELISpot was a sensitive assay to detect β -lactam-induced skin injury.

In agreement with the genetic association study of Daly et al, 4 patients with a positive flucloxacillin PBMC ELISpot expressed HLA-B*57:01. The majority of flucloxacillin clones isolated from these patients were CD8+ and presentation of the drug-derived antigen was HLA-class I restricted. Utilizing antigen presenting cells from blood donors expressing different HLA-B alleles it was possible to show that these clones were activated with flucloxacillin-pulsed antigen presenting cells only if the cells expressed B*57:01. These data are the first to fully characterize the nature of the drug-specific T-cell response in patients with liver injury. In on-going experiments, we have now isolated and are beginning to characterize drug-specific T-cells from patients with co-amoxiclav, isoniazid and trimethoprim-induced liver

injury. Thus, in years to come it is highly likely that the adaptive immune system is shown to play an important role in liver reactions to numerous drugs.

Next, we utilised our HLA-typed cell bank containing PBMC from 400 HLA-typed (drug naïve) blood donors (Alfirevic et al., 2012) and a recently established dendritic cell T-cell priming assay (Faulkner et al., 2012a) to study whether flucloxacillin activates naïve T-cells in an HLA-B*57:01 restricted fashion. This assay relies on the isolation and culture of highly pure T-cell and antigen presenting cell populations. Immature monocyte-derived dendritic cells and naïve T-cells are used as antigen presenting cells and responder cells, respectively. After an 8 day culture period, T-cells are re-exposed to flucloxacillin and dendritic cells and antigen specificity is measured shortly after. Through cloning of the drug primed T-cell cultures, flucloxacillin was shown to selectively activate CD8⁺ T-cells and again the T-cell response was HLA-B*57:01 restricted. Collectively, these studies link the genetic association to the disease pathogenesis. The one area that remains to be addressed is to investigate how and why flucloxacillin-specific T-cells kill liver cells in susceptible patients. A major obstacle to these studies is a lack of *autologous* immune and liver cells. On-going studies in Liverpool we are attempting to overcome this through the generation of HLA-matched induced pluripotent stem cell-derived hepatocyte-like cells.

Utilising the same approach as described above with piperacillin we found that the T-cell response to flucloxacillin was concentration and time-dependent, which suggests that a protein adduct is needed to activate clones. Interestingly, in contrast to the piperacillin clones, flucloxacillin clones were broadly crossreactive. They were activated by a variety of β -lactam antibiotics, indicating that the core β -lactam ring structure is the main chemical component involved in the MHC T-cell receptor binding interaction [Figure 6.1].

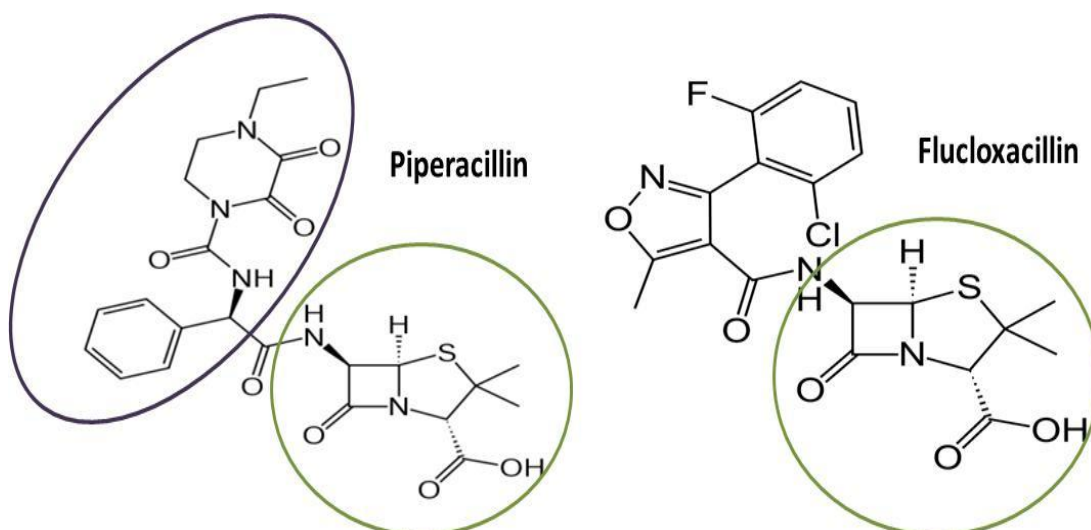


Figure 6.1- Illustration of chemical structure of piperacillin and flucloxacillin.

Studies originating after our findings were published (Monshi et al., 2013) from the group of Pichler (Wuillemin et al., 2013) using clones from generated from drug-naïve volunteers found a similar hapten response. However, they also found that certain clones were activated rapidly, with flucloxacillin binding directly to MHC associated peptides. Whether this binding involves a covalent or non-covalent interaction with specific amino acid residues is still open to debate. In fact, it is feasible that certain drug-responsive clones are activated via direct (covalent and non-covalent) modification of MHC binding peptides and flucloxacillin protein adducts following processing.

What we know and don't know!

Piperacillin

- ▶ Clinical features
 - 12g per day (in CF)
 - iv
 - *Skin reaction*
(1/3 patients)
- ▶ Mechanistic features
 - Antigen in patients
(*lysosomal processing of plasma albumin?*)
 - No known HLA restriction
 - T-cell (CD4+)
 - No cross-reactivity

Flucloxacillin

- ▶ Clinical features
 - 1g per day
 - oral
 - *Liver reaction*
(1/10000 patients)
- ▶ Mechanistic features
 - Antigen in patients
(*proteasomal processing of liver albumin?*)
 - B*5701 restricted
 - T-cell (CD8+)
 - Broadly cross-reactive

Why organ selectivity ?
Why the difference in frequency ?

Figure 6.2- The clinical and mechanistic basis of piperacillin and flucloxacillin hypersensitivity. Taken from (Uetrecht and Naisbitt, 2013).

The reason why these two β -lactam antibiotics piperacillin and flucloxacillin selectively target the skin and liver is still not fully understood. Furthermore, the reason for the difference in reaction frequency is not known. Figure 6.2 summarises the key clinical and mechanistic features of these two forms of drug hypersensitivity. If one first considers piperacillin-induced skin injury. This drug is administered at high doses and patients receive repeated courses (up to 4 per year). This equates to around 6.5kg of piperacillin over a 10 year period. This incredible drug burden coupled with the immune dysfunction associated with cystic fibrosis and repeated cycle of infection and inflammation could account for the high incidence of cutaneous reactions. Koch et al (Koch et al., 1991) found that 4.5% of 2793 β -lactam treatment courses resulted in an adverse reaction. Wills (Wills et al., 1998) found similar results, a retrospective analysis of 53 patients with cystic fibrosis identified hypersensitivity reactions in 34% of individuals. In the Leeds cystic fibrosis cohort, the data are similar; an analysis in 2011 identified 302 β -lactam reactions in 375

patients. Of particular interest is why piperacillin selectively activates CD4⁺ T-cells. Is it possible that piperacillin albumin adducts, represent the primary antigen that promotes reactions in patients? Human serum albumin is known to account for the majority of serum-bound penicilloyl groups in patients (Lafaye and Lapresle, 1988) and cell assays (Whitaker et al., 2011a; El-Ghaiesh et al., 2012) and such exogenous protein adducts would be preferentially processed via the lysosomal pathway generating MHC class II binding peptides. If this scenario is correct, one would still need to explain why flucloxacillin, which also binds selectively to albumin lysine residues preferentially activates CD8⁺ T-cells and causes liver injury. Hepatocytes are the bodies' main producer of albumin. Thus, one possibility that the group in Liverpool group is working on is that flucloxacillin selectively binds to hepatocyte albumin generating an intracellular protein adduct that would be processed via a proteosomal pathway generating MHC class I binding peptides. Flucloxacillin is a potent inhibitor of the bile salt export pump (a membrane protein localized in the cholesterol-rich canalicular membrane of hepatocytes, which shows that it enters hepatocytes (Thompson et al., 2012; Warner et al., 2012). Furthermore, Carey et al. (Carey and van Pelt, 2005) identified a small number of drug-modified liver proteins (including albumin) in flucloxacillin-exposed rats using Western blot analysis. However, experiments to assess drug protein binding in human hepatocytes have not been performed; thus, the relationship between antigen formation in target tissue and HLA-restricted killing by cytotoxic T-cells has not been defined.

Differential distribution of drug protein binding represents one possible explanation as to why piperacillin and flucloxacillin cause drug-induced skin and liver injury, respectively. Other possible explanations include concomitant disease, which may regulate co-stimulatory signalling in different tissues. It is also possible that the site of T-cell priming has little bearing on the nature of the tissue injury and that the homing receptors expressed on the surface of drug-specific T-cells is the primary determinant. In this respect, we have shown that piperacillin and flucloxacillin-specific T-cell express high levels of skin-homing and gut-homing chemokine receptors, respectively. However, much more work is clearly needed to

elucidate the reasons why such structurally similar drugs cause different forms of adverse drug reaction.

In conclusion, the aim of this thesis was to further our understanding of the chemical and cellular basis of β -lactam hypersensitivity reactions. I have been successful in both respects, however, research in this area must continue if we are to understand fully why β -lactam antibiotics cause immune-mediated adverse effects in susceptible patients and develop models for the pharmaceutical industry to assist the development of safer drugs.

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